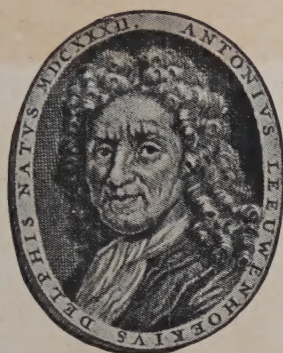


# ANTONIE VAN LEEUWENHOEK

Journal of Microbiology and Serology



Official publication of the  
„NEDERLANDSCHE VEREENIGING VOOR MICROBIOLOGIE”  
(Netherlands Society of Microbiology)

---

Board of Editors:

A. CLARENBURG, Utrecht; H. W. JULIUS, Utrecht;  
T. Y. KINGMA BOLTJES, Amsterdam; A. J. KLUYVER,  
Delft; A. CHARL. RUYS, Amsterdam; JAN SMIT, Wage-  
ningen; E. P. SNIJDERS, Amsterdam; J. D. VERLINDE,  
Leiden; JOHA. WESTERDIJK, Baarn; K. C. WINKLER,  
Utrecht.

---

Editors:

MARIE P. LÖHNIS, Wageningen  
L. H. C. PERQUIN, Breda

---

SWETS EN ZEITLINGER  
BOEKHANDEL EN UITGEVERSMAATSCHAPPIJ — AMSTERDAM

|                                                                                                                                                                              | Page |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| AUDIER, A. G., Chemotherapy of carcinoma improved by host conditioning. II. Local chemotherapy combined with indirect treatment                                              | 201  |
| BEUMER, J. et VANDEWEYER-DEVEEN, A., Premiers résultats de la lysotypie de <i>S. paratyphi</i> B en Belgique . . . . .                                                       | 263  |
| VAN BEVERWIJK, AGATHE L., Three new fungi: <i>Helicoon pluriseptatum</i> , <i>Papulaspora pulmonaria</i> , and <i>Tricellula inaequalis</i> . . . . .                        | 1    |
| BEZJAK, V., Differentiation of <i>Corynebacterium diphtheriae</i> of the <i>mitis</i> type found in diphtheria and ozaena. I. Biochemical properties. . .                    | 269  |
| BONNIER, CH. s. HELY, F. W.                                                                                                                                                  |      |
| BOTS, L. s. VAN DER VEEN, J.                                                                                                                                                 |      |
| BREED, R. S. and LESSEL JR., E. F., The classification of luminescent bacteria . . . . .                                                                                     | 58   |
| DE BRUIN, A. S. s. MOSSEL, D. A. A.                                                                                                                                          |      |
| CAPRIOTTI, A., Recherches sur les levures de la fermentation vinaire en Italie. (Avec une note sur les levures du moût de raisin „Frankenthaler” cultivé en serre) . . . . . | 374  |
| CHOW, K. Y. s. SIMONART, P.                                                                                                                                                  |      |
| CLARENBURG, A., MARSEILLE, A. and VINK, H. H., A newly discovered <i>Salmonella</i> type ( <i>S. israel</i> ) . . . . .                                                      | 319  |
| CLARENBURG, A., TEUNISSEN, G. H. B. and VAN DER VLERK, J. <i>Salmonella israel</i> in a dog . . . . .                                                                        | 415  |
| COULON, T. s. FRATEUR, J.                                                                                                                                                    |      |
| FRATEUR, J., SIMONART, P. et COULON, T., Étude chromatographique de cultures d' <i>Acetobacter</i> . . . . .                                                                 | 111  |
| GODIN, P., Chromatographie des composants ternaires du mycélium de <i>Penicillium brevi-compactum</i> . . . . .                                                              | 321  |
| GOOS, J. J. C. s. VERHOEVEN, W.                                                                                                                                              |      |
| HANSEN, P. A. s. LANGFORD JR., G. C.                                                                                                                                         |      |
| HAUDUROY, P. s. VAGO, C.                                                                                                                                                     |      |
| HELY, F. W. and BONNIER, CH., The effects of induced soil aggregation on the occurrence of <i>Azotobacter</i> and <i>Streptomyces</i> . . . . .                              | 359  |
| HERTZBERGER, E., Sterility test on water oil emulsions. . . . .                                                                                                              | 167  |
| HEYL, J. G., On the occurrence and the significance of paracolon bacteria in the Netherlands . . . . .                                                                       | 406  |
| KLUYVER, A. J. and VERHOEVEN, W., Studies on true dissimilatory nitrate reduction. II. The mechanism of denitrification . . . . .                                            | 241  |
| KLUYVER, A. J. and VERHOEVEN, W., Studies on true dissimilatory nitrate reduction IV. On adaptation in <i>Micrococcus denitrificans</i> . .                                  | 337  |
| KOSTER, A. J. s. VERHOEVEN, W.                                                                                                                                               |      |
| LANGFORD JR., G. C. and HANSEN, P. A., The species of <i>Erysipelothrix</i>                                                                                                  | 87   |
| LEIFSON, E., The flagellation and taxonomy of species of <i>Acetobacter</i> .                                                                                                | 102  |



|                                                                                                                                                                                                                         |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| LESSEL JR., E. F. S. BREED, R. S.                                                                                                                                                                                       |     |
| LIE, K. T. S. VERLINDE, J. D.                                                                                                                                                                                           |     |
| LINDENBERGH, D. J. S. VAN SCHREVEN, D. A.                                                                                                                                                                               |     |
| LOLKEMA, H., Acid production by <i>Sc. thermophilus</i> . . . . .                                                                                                                                                       | 153 |
| MACKINNEY, G. S. NAKAYAMA, T.                                                                                                                                                                                           |     |
| MAKI, L. R., Experiments on the microbiology of cellulose decomposition<br>in a municipal sewage plant . . . . .                                                                                                        | 185 |
| MARSEILLE, A. S. CLARENBURG, A.                                                                                                                                                                                         |     |
| MOLRON, J. H., Le phénomène du double anneau dans l'étude des<br>phagostatiques . . . . .                                                                                                                               | 329 |
| MOLRON, J. H. S. VERLINDE, J. D.                                                                                                                                                                                        |     |
| MOSSEL, D. A. A. and DE BRUIN, A. S., A gelatin liquefaction test for<br>the screening of compounds used or proposed as inhibitors of bacterial<br>proteolytic deterioration in foods . . . . .                         | 233 |
| NAKAYAMA, T., MACKINNEY, G. and PHAFF, H. J., Carotenoids in<br>asporogenous yeasts . . . . .                                                                                                                           | 217 |
| NAUTA, W. TH. S. VAN DER STELT, C.                                                                                                                                                                                      |     |
| VAN NIEVELT, M. C. A. S. VERHOEVEN, W.                                                                                                                                                                                  |     |
| OTZEN, D. S. VAN SCHREVEN, D. A.                                                                                                                                                                                        |     |
| PHAFF, H. J. S. NAKAYAMA, T.                                                                                                                                                                                            |     |
| VAN RAMSHORST, J. D. and RIJKS, H., The flocculation of tetanus toxin<br>and toxoid. I. . . . .                                                                                                                         | 17  |
| RANGANAYAKI, S., A study of the influence of ammonium sulphate on<br>the mineral intake of <i>Pichia membranaefaciens</i> at different pH. . .                                                                          | 429 |
| RICHARD, O. S. WIKÉN, T.                                                                                                                                                                                                |     |
| RUYS, A. CH., Inhibition of growth of tubercle bacilli by ali-esterase<br>inhibitors in various culture media. . . . .                                                                                                  | 145 |
| RIJKS, H. S. VAN RAMSHORST, J. D.                                                                                                                                                                                       |     |
| VAN SCHREVEN, D. A., OTZEN, D. and LINDENBERGH, D. J., On the<br>production of legume inoculants in a mixture of peat and soil . . .                                                                                    | 33  |
| SIMONART, P. et CHOW, K. Y., Étude du métabolisme d'acides aminés<br>chez <i>Aspergillus oryzae</i> . III. Acides aminés libres dans le mycélium<br>cultivé sur diverses sources de carbone en présence d'ammoniaque. . | 174 |
| SIMONART, P. et CHOW, K. Y., Étude du métabolisme d'acides aminés<br>chez <i>Aspergillus oryzae</i> . IV. Influence du chlorure de calcium sur le<br>métabolisme des acides aminés . . . . .                            | 210 |
| SIMONART, P. S. FRATEUR, J.                                                                                                                                                                                             |     |
| SMITH, L. S. TASMAN, A.                                                                                                                                                                                                 |     |
| VAN DER STELT, C., ZWART VOORSPUIJ, A. J. and NAUTA, W. TH.,<br>Investigation into the tuberculostatic activity of some derivatives<br>of p. aminobenzoic acid . . . . .                                                | 285 |
| STOLK, A. C., <i>Aspergillus asperescens</i> n. sp. . . . .                                                                                                                                                             | 299 |

|                                                                                                                                                                                                                                                 | Page |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| TASMAN, A. and SMITH, L. On the pathogenesis of diphtheria. III.<br>On the formation of diphtheria toxin in fresh human saliva. . . .                                                                                                           | 417  |
| TEUNISSEN, G. H. B. s. CLARENBURG, A.                                                                                                                                                                                                           |      |
| VAN TONGEREN, H. A. E., Biochemical properties and the production<br>of toxins and enzymes by Staphylococci isolated from cases of<br>secondary pneumonia following influenza. . . . .                                                          | 65   |
| VAN TONGEREN, H. A. E., The isolation and some characteristics of<br>bacteriophages from Staphylococci present in cases of secondary<br>pneumonia following influenza. . . . .                                                                  | 81   |
| VAGO, C. et HAUDUROY, P., Résistance à la lyophilisation, du virus de<br>la polyédrée de <i>Thaumetopoea pityocampa</i> Schiff (Lepidoptera). . .                                                                                               | 141  |
| VANDEWEYER-DEVEEN, A. s. BEUMER, J.                                                                                                                                                                                                             |      |
| VAN DER VEEN, J. and BOTS, L., A simple method for determining the<br>sensitivity of micro-organisms to serial dilutions of antibiotics. . .                                                                                                    | 229  |
| VERHOEVEN, W. and GOOS, J. J. C., Studies on true dissimilatory<br>nitrate reduction. I. Fate of the hydrogen donator in bacterial<br>nitrate reduction. . . . .                                                                                | 93   |
| VERHOEVEN, W., KOSTER, A. J. and VAN NIEVELT, M. C. A., Studies on<br>true dissimilatory nitrate reduction. III. <i>Micrococcus denitrificans</i><br>Beijerinck, a bacterium capable of using molecular hydrogen in<br>denitrification. . . . . | 273  |
| VERHOEVEN, W. s. KLUYVER, A. J.                                                                                                                                                                                                                 |      |
| VERLINDE, J. D. and MOLRON, J. H., Mixed infection with type 2<br>poliomyelitis virus and Colombia SK group virus in man. An attempt<br>to explain any relation of viruses of the Columbia SK group to human<br>poliomyelitis. . . . .          | 129  |
| VERLINDE, J. D. and LIE, K. T., Experimental infection of suckling-<br>ferrets with viruses of the Coxsackie group isolated from cases of<br>infantile gastroenteritis. . . . .                                                                 | 181  |
| VINK, H. H. s. CLARENBURG, A.                                                                                                                                                                                                                   |      |
| VAN DER VLERK, J. s. CLARENBURG, A.                                                                                                                                                                                                             |      |
| VOORHORST, R., The counting of viable tubercle bacteria. . . . .                                                                                                                                                                                | 305  |
| WIKÉN, T. und RICHARD, O., Untersuchungen über die Physiologie der<br>Weinhefen. V. Mitteilung. Über die Kohlendioxydproduktion der<br>„Fendant“-Weinhefe in Stickstoff bzw. Argon, Luft und Stickstoff-<br>bzw. Argon-Luft-Gemisch. . . . .    | 385  |
| ZWART VOORSPIJ, A. J., Influence de l'action combinée du P.A.S. et<br>de l'hydrazide isonicotinique (INH) sur l'augmentation de la résis-<br>tance de bacilles tuberculeux in vitro. . . . .                                                    | 315  |
| ZWART VOORSPIJ, A. J. s. VAN DER STELT, C.                                                                                                                                                                                                      |      |



(Centraal Bureau voor Schimmelcultures, Baarn, Netherlands).

THREE NEW FUNGI:  
*HELICOON PLURISEPTATUM* n.sp.  
*PAPULASPORA PULMONARIA* n.sp.  
and *TRICELLULA INAEQUALIS* n.gen., n.sp.

by

AGATHE L. VAN BEVERWIJK

(Received May 13, 1953).

The purpose of this paper is to describe three fungi that are believed to be new to science. Two fungi were encountered in an investigation of moulds growing on decaying leaves, the third fungus was sent for identification to the Centraal Bureau voor Schimmelcultures. Living cultures of these species are maintained at the C.B.S. Herbarium material of *Helicoon pluriseptatum* and *Papulaspora pulmonaria* will be deposited at the Herbarium of the Royal Botanic Gardens at Kew. Dried authentic cultures will be sent to the Rijksherbarium at Leiden, Netherlands, to the Kew Herbarium and to the herbarium of the Commonwealth Mycological Institute, Kew, England.

*Helicoon pluriseptatum* n.sp.

This fungus with beautiful spiral spores was found growing on decaying leaves collected from fens and marshy places in woods in the Netherlands. In some fens near Apeldoorn it is quite frequent, almost every collection of blackened birch leaves yields this species. Further the fungus was collected from pine needles, a pine cone, from leaves of red oak and from blades of grass. A collection made from birch leaves found in a fen near Emst (Veluwe), August 1951, is considered as the type of this species.

On the leaves the colony is inconspicuous. The fungus is detected by its spores which, when observed with a dissecting microscope, appear singly or in groups of two or three, just above the water

film adhering to the leaf. The mature spores are easily shed and float off at the slightest disturbance. Only in carefully handled Necol mounts (ELLIS, 1950), spores are found still attached to their conidiophores. The mycelium grows in the decaying plant material. The conidiophores arising from this mycelium are very variable in length, 25–320  $\mu$  and longer; they may be simple and short (Fig. 1 a–c), or irregularly branched (Fig. 2), or very long and not different

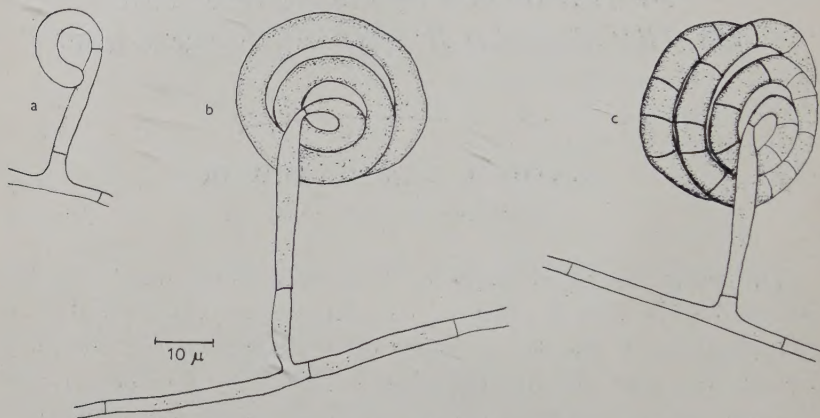


Fig. 1. *Helicoon pluriseptatum*.

- a. Conidiophore with developing conidium.
- b. Conidiophore with young conidium coiling away from the observer. Septa not yet formed.
- c. Nearly mature conidium still attached to the conidiophore.

from the ordinary hyphae. The width of the conidiophore, like that of the hyphae, measures 3  $\mu$ ; the wall at the base is not thickened as it is in *Helicoon fuscosporum* Linder (VAN BEVERWIJK, 1953). When one to several septa have been formed, the tip, distal from the final septum, begins to coil to form the conidium, the spore filament soon broadens to 5  $\mu$  (Fig. 1 a–c), tapering again to 2.5  $\mu$  in the top coil. The developing spore is light fuscous and has but few septa (Fig. 2). The mature spore is dark fuscous and has many conspicuous cross-walls (Figs. 3, 4b); in a spore with 3½ coils, 43 cross-walls were counted. The top and bottom of the spore remain rather flat, this gives the spore the shape of a tangerine (Fig. 4a). The direction of the coiling is constantly counter clockwise (DAVIDSON and GREGORY, 1938) (Fig. 4c).

**Cultures:** several strains of this fungus were isolated in pure



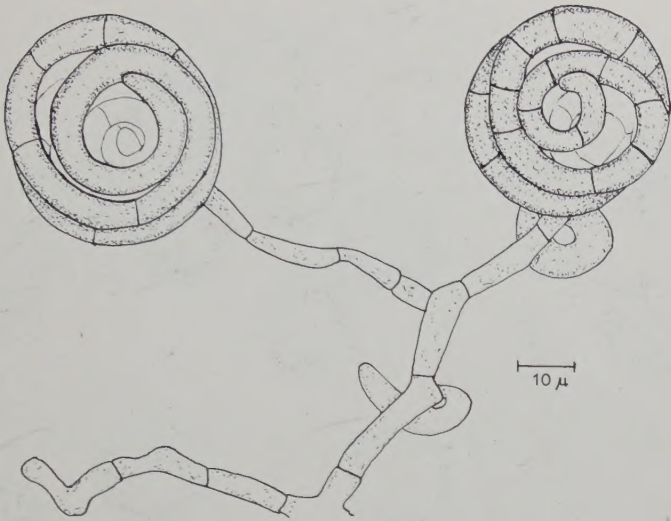


Fig. 2. *Helicoon pluriseptatum*.

Branched conidiophore with nearly mature conidium on the right side and developing conidium on the left, septa still scarce, especially in the top coils. Necol mount from decaying birch leaves.

culture. The spores germinate readily, producing germ tubes from several cells of the spiral, these germ tubes have about the same diameter and colour as the ordinary hyphae. The fungus grows well on most culture media. On cherry agar plate at room temperature the culture reaches a diameter of 5—6 cm in 4 weeks. The texture of the colony is woolly (LONG and HARSCH, 1918), the margin somewhat uneven. The aerial mycelium is greyish when young, turning brownish grey when aging, the reverse of the colony is black. Spores are produced on the aerial mycelium. In culture the conidiophores are almost always long, unbranched and not differentiated, bearing a single terminal conidium. Observations on a developing spore showed that it takes about eight hours to complete one coil.

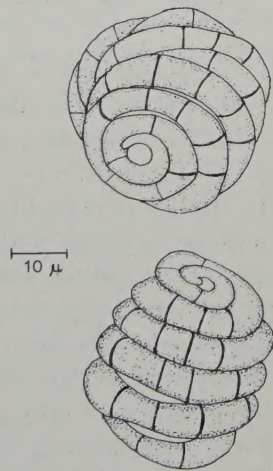


Fig. 3. *Helicoon pluriseptatum*. Mature conidia.

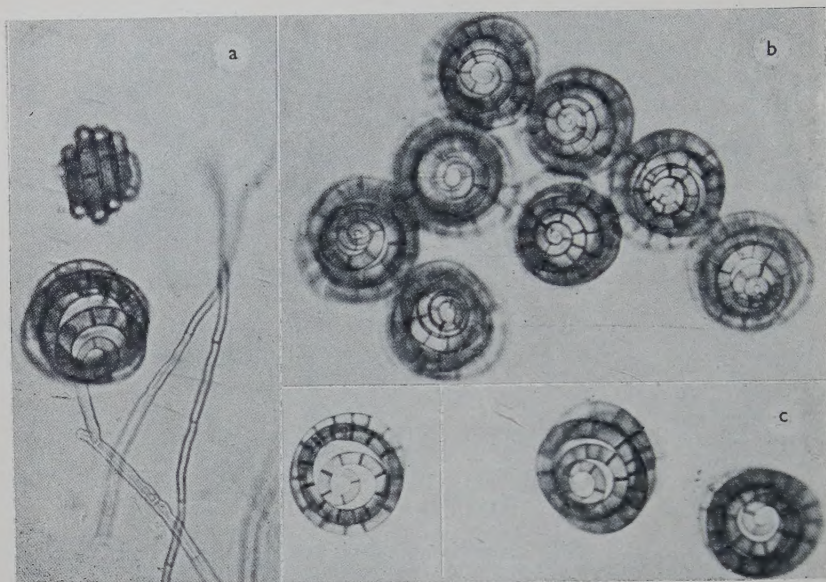


Fig. 4. *Helicoon pluriseptatum*.

- a. Conidiophore with conidium seen from the bottom. A detached conidium seen in side view.  $\times 390$ .
- b. Mature conidia, focused on the middle coils, to show the many cross-walls.  $\times 390$ .
- c. Detached conidia, focused on top coils, to show the counter clockwise coiling.  $\times 390$ . From decaying birch leaves.

Good sporulation is obtained on cherry agar and sterilized lupin stem. Exposure to the light does not seem to be as necessary a factor for sporulation as it is in many of the *Helicosporae*.

This fungus can best be placed in the genus *Helicoon* Morgan of the Helicosporous Fungi Imperfecti. The present species differs from the nearly related *H. fuscosporum* Linder by the size and shape of its spore and the number of cross-walls in the spore filament. The fungus was also compared with the description of SCHULZER's *Helicosporium politulum*. LINDER (1929) considered this species as an imperfectly known *Helicoon*. The conidiophores which SCHULZER (1877) describes grow densely and are unbranched: "... "ein Wald etwas weitschichtig septirter an den Theilungstellen hie und da etwas eingeschnürter einfacher Sporenträger". These conidiophores do not agree very well with those observed on the present species. As we may gather from SCHULZER's rather vague description, the



conidiophore develops a single terminal spiral spore, coiled from the right to the left. Unfortunately SCHULZER does not give any measurements or figures, either of the conidia or of the conidiophores. Although the fungus under consideration seems related to SCHULZER's species, it will never be possible to identify any *Helicosporous* fungus with this scanty description lacking essential data. Therefore it seems best that the present fungus should be considered as a new species of the genus *Helicoon*.

*Helicoon pluriseptatum* n.sp.

Aero-aquatic fungus with branched septate fuscous mycelium. Conidiophores may be short, simple or sparingly branched; or very long and not different from the ordinary hyphae,  $3\ \mu$  wide. Conidia fuscous, diameter  $31-45\ \mu$ ,  $23-30\ \mu$  high, filament  $5\ (6)\ \mu$ , coiled in three planes, to form a blunt oval spore body,  $5-6\ (4-7)$  seriate,  $12-17$  septa per coil. Direction of coiling counter clockwise.

Hab. on decaying plant material in water. Netherlands.

*Helicoon pluriseptatum* sp.nov.

Fungus aëro-aquaticus mycelio ramoso, septato, fusco. Conidiophora aut brevia, simplicia vel parce ramosa, aut longissima, non distincta a hyphis communibus,  $3\ \mu$  crassa. Conidia fusca, diametro  $31-45\ \mu$ , alta  $23-30\ \mu$ . Fila  $5\ (6)\ \mu$  lata, convoluta in tribus planis, ut formetur corpus ovatum,  $5-6\ (4-7)$  vicibus, septis  $12-17$  in singulis spiris. Fila voluntur contrarie atque indicis horologii hodierni. Hab. In membris plantarum sub aqua putrescentibus. Hollandia.

*Papulaspora pulmonaria* n.sp.

From an old tin filled with rain water in a garden at Baarn some decaying leaves were taken to the laboratory. On this plant material, mainly blackened beech leaves, the present fungus was found growing, January 1951 (typus). The fungus was collected a second time from this tin in November 1952. Another strain of the same fungus was obtained from Miss GLEN-BOTT<sup>1)</sup> who isolated it from a blackened willow leaf in a pond near Swanley, England, April 1950.

<sup>1)</sup> The author wishes to express her sincere thanks to Miss GLEN-BOTT for providing her with this isolate.

The collected leaves were kept damp in Petri dishes; after several weeks a group of small, grey-black granules became visible. No mycelium was seen. The growth was rather scanty. With the aid of a sterile glass needle some granules were transferred to a suitable medium in which they germinated readily, sending out many germ tubes. At first sight these granules rather resemble the spores of *Sporidesmium* Link. Observations on the development of these reproductive bodies, however, show that they cannot be considered as dictyospores, as they are not the result of division of a single cell, but originate from many-celled primordia. The reproductive bodies of this fungus can best be regarded as bulbils. Imperfect fungi with similar reproduction were united by HOTSON in the form-genus *Papulaspora* Preuss. In his monograph on these fungi HOTSON (1912) repeatedly stresses the fact that bulbil producing forms are developed "by a variety of species wholly unrelated and belonging to widely separated groups among the Pyrenomycetes, the Disco-mycetes and the Basidiomycetes" (l.c. page 301). In the genus *Papulaspora* are placed all those bulbiferous fungi of which the perfect form is not known. It is not surprising that this heterogenous form-genus shows many morphological differences in the development of the bulbil. HOTSON distinguishes three groups of bulbils, according to the development of the primordium (l.c. page 273): "those which form an intercalary primordium of several cells, those which typically originate from a primary spiral; and those that are produced by a perpendicular branch or branches which do not form a spiral". In the present species the bulbil develops from a branched primordium which terminates a pedicel arising from the submerged mycelium.

**Cultures:** the fungus grows well on a number of different media, the best development of bulbils was obtained on cherry agar, cherry agar mixed with a decoction of horse dung, on sterilized lupin stem and on Lutz agar. The rate of growth is not very fast, on cherry agar plate at room temperature the culture reaches a diameter of 4 cm after one month. The aerial mycelium is grey, woolly, hyphae 2—2.5  $\mu$  wide, the substrate mycelium is black with a dark greenish tinge. Bulbils are produced in those parts of the culture where little aerial mycelium develops. From the blackish submerged mycelium stalks arise of varying length, generally one to three times as long as the bulbil. The width of these slightly tapering pedicels is 4—6  $\mu$  at the base; one to several, mostly three



to four, septa develop. Near the apex of the stalk one or two branches may grow out, which form the ground pattern of the bulbil (Fig. 5*a* and *b*). At right angles from the main axis and the branches, short side branches grow out which divide dichotomously (Figs. 5*b* and *c*, 6). This dichotomous division is repeated several times, resulting in a number of short septate branches (Fig. 5*c*). The final divisions of these branches widen to spherical cells, not unlike alveoli

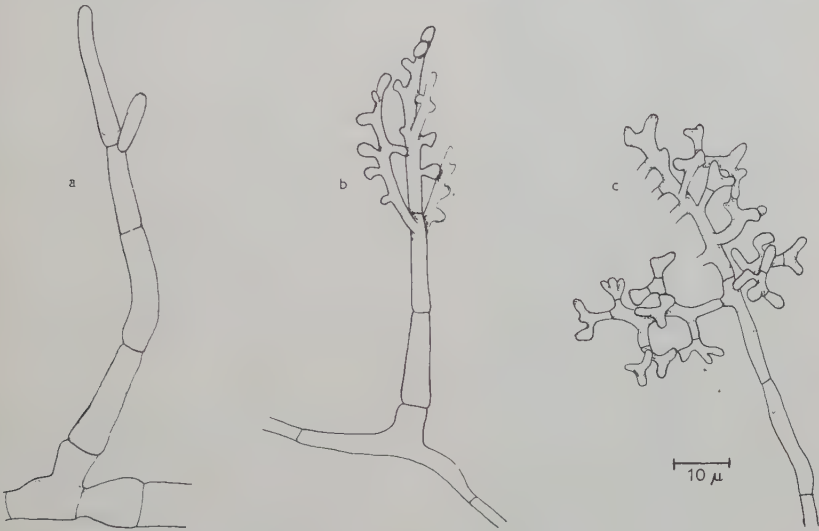


Fig. 5. *a—c. Papulaspora pulmonaria.*

Developing primordia. From agar culture. Necol mounts in lactic acid with cotton blue.

terminating the bronchioli in the lungs of mammals. Air remains caught between the branches. When still hyaline or light fuscous bulbils are mounted in lactic acid, most of the air is dispelled and the system of branches inside can be observed, when focusing on the interior of the bulbil (Fig. 7*a* and *b*). Microtome sections confirmed what was seen in optical section, moreover they showed that the surface of the bulbil consists of only one layer of spherical cells packed closely together and becoming angular by mutual pressure. Through the hollow interior of the bulbil an intricate system of septate branches stretches, each branch ending in a spherical cell (Figs. 8, 9). Young bulbils are hyaline, in reflected light they appear glistening white. Even in cultures of several months old, some white bulbils are seen, scattered between the dark mature ones, which are

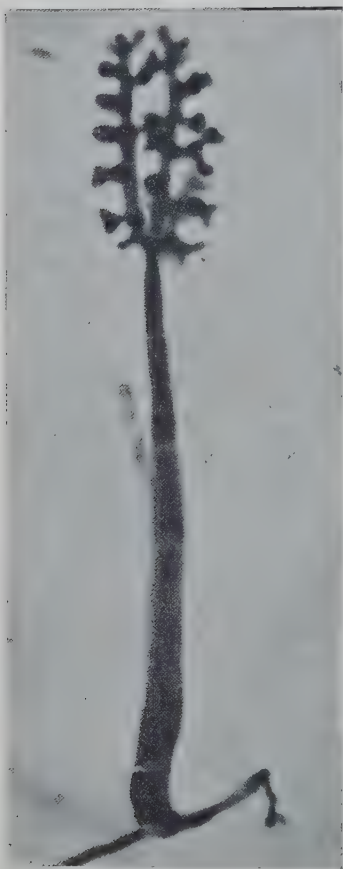


Fig. 6. *Papulaspora pulmonaria*. Young primordium. From agar culture, mounted in lactic acid with cotton blue.  $\times 970$ .

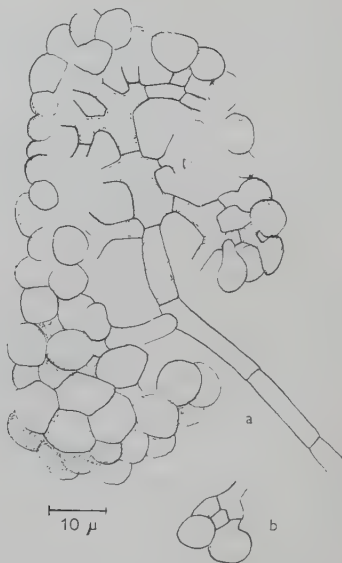


Fig. 7. *Papulaspora pulmonaria*.  
 a. Young bulbil focused on the interior, to show branching system and spherical cells terminating the dichotomous divisions. Left-hand side lower down, focused on the exterior, to show surface of the bulbil.  
 b. Detail from a, showing final dichotomous divisions widening to spherical cells. From agar culture, mounted in lactic acid with cotton blue.

black in reflected light, dark fuscous in transmitted light. The mature bulbils are irregularly lobed, in mounts they are mostly seen in side view and then often somewhat resemble young ivy leaves (Figs. 10, 11). By rolling over the bulbils between slide and coverglass, it appeared that they were not tetrahedral in shape as might be supposed, but nearly always flattened. The measurements of the bulbils are: 62—110  $\mu$  long, 57—103  $\mu$  broad, 35—55  $\mu$  thick. In the woolly aerial mycelium there is the same tendency





Fig. 8. *Papulaspora pulmonaria*.  
Microtome section of mature  
bulbil.

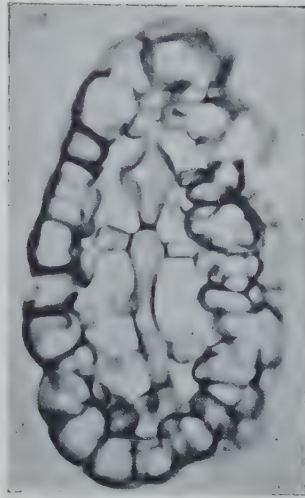


Fig. 9. *Papulaspora pulmonaria*.  
Microtome section of mature  
bulbil.  $\times 680$ .

to form side branches with dichotomous divisions (Fig. 12a) and spherical cells (Fig. 12b). These may develop either intercalary or terminally, but no typical bulbils result from these structures, only rather loosely aggregated masses of globular cells are formed by a process of gemmation (Fig. 12c). No other spore form has been observed. Three monosporic isolates were paired with each other and with the strain obtained from Miss GLEN-BOTT, but no perfect form has developed in these cultures. Of all described species of bulbiferous fungi, *Papulaspora pallidula* Hotson is the only species which shows a primordium formed by dichotomously dividing branches (HOTSON, 1917). As may be gathered however from HOTSON's figures (l.c. Figs. 2—8) this primordium resembles the more simple structures such as develop on the aerial mycelium of the fungus under consideration. The termi-

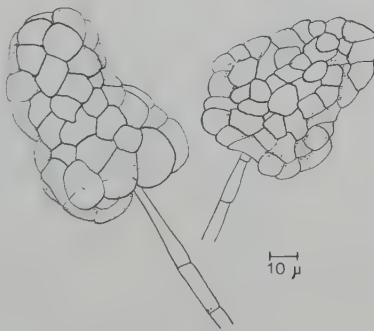


Fig. 10. *Papulaspora pulmonaria*.  
Mature bulbils. From Lutz agar  
culture, one month old; mounted  
in lactic acid.

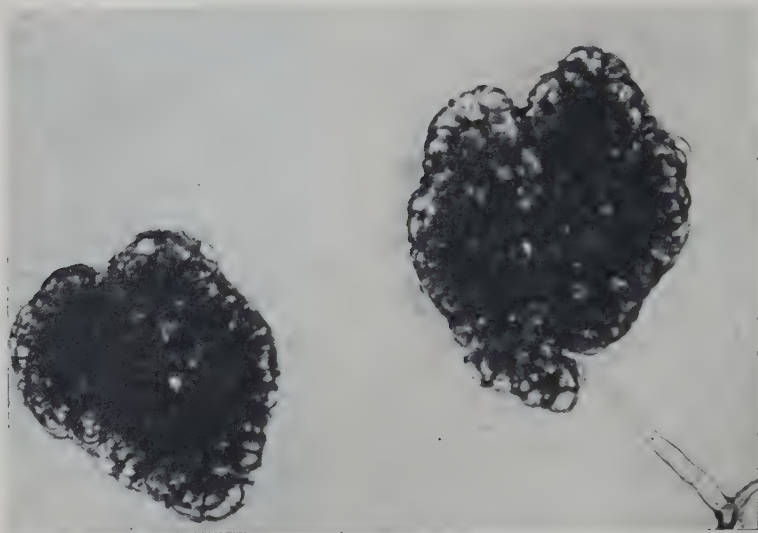


Fig. 11. *Papulaspora pulmonaria*.  
Mature bulbils. From culture, mounted in lactic acid.  $\times 440$ .

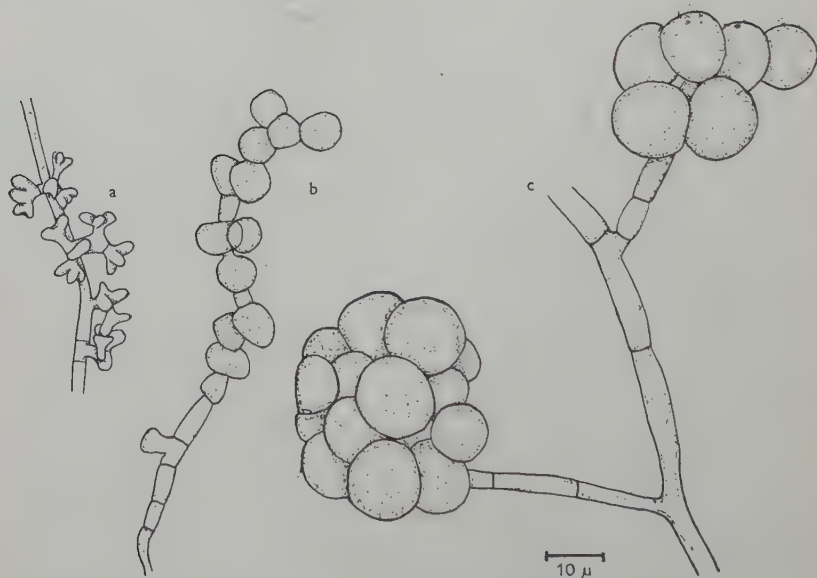


Fig. 12. *Papulaspora pulmonaria*.  
Bulbilliform structures on the aerial mycelium. From culture.  
a. Intercalary dichotomous divisions.  
b. Spherical cells.  
c. Aggregates of spherical cells.

nal, much branched, primordia formed on well developed pedicels as observed on the present species seem to be a new mode of development in this already very varied genus.

The name *Papulaspora pulmonaria* is suggested for this fungus, because the formation of the bulbil reminds one of the microscopic structure of lungs.

*Papulaspora pulmonaria* n.sp.

Mycelium septate, branched, fuscous. Bulbils dark fuscous, irregularly lobed, somewhat flattened, 62—110  $\mu$  long, 57—103  $\mu$  broad, 35—55  $\mu$  thick, developing on pedicels, 80—300  $\mu$  long, 3—4  $\mu$  wide at base. Primordium, terminating the pedicel, consists of one to three branches forming septate side branches by dichotomous division, the final divisions widen to spherical cells, which constitute the bulbil.

Hab. On decaying leaves in water. Netherlands, England.

*Papulaspora pulmonaria* sp.nov.

Mycelium septatum, ramosum, fuscum. Bulbuli atro-fusci, varie lobati, paulum planati, 62—110  $\mu$  longi, 57—100  $\mu$  lati, 35—55  $\mu$  crassi, orti e pediculis, 80—300  $\mu$  longis, prope basim 3—4  $\mu$  crassis. Primordium pediculum terminans constat e maxime tribus ramis, e quibus rami laterales septati oriuntur, qui per dichotomiam alios ramos efficiunt, qui eodem modo alios procreant. Extremae partes ramorum latescunt in cellulas rotundas, quae bulbulum formant.

Hab. In foliis sub aqua putrescentibus. Hollandia, Anglia.

*Tricellula inaequalis* n.gen., n.sp.

This fungus was sent to the C.B.S. for identification by Dr EILERT EILERTSEN who isolated it from the air in the course of a study of fungus allergy. Dr EILERTSEN kindly gave us the following details about his isolate. "The fungus Nr 143 was isolated from an agar plate exposed for 15 minutes in an open square outside the university of Bergen (Norway). For a week there had been a layer of 10 cm of snow. The

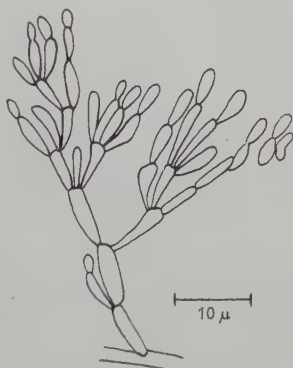


Fig. 13.

*Tricellula inaequalis*.

Compound conidiophore with liberated conidium. From a slide culture, mounted in lactic acid with cotton blue.



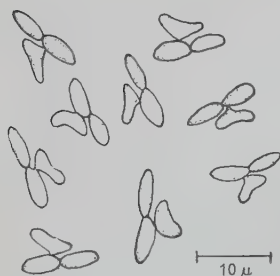


Fig. 14.

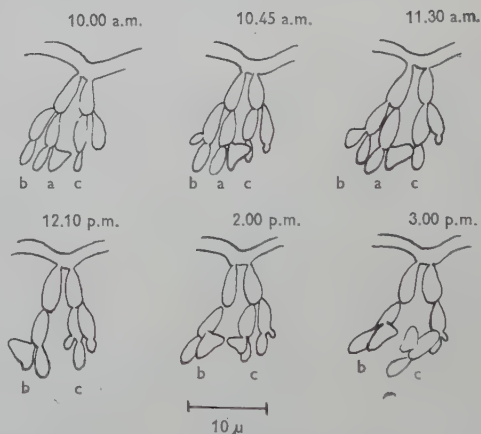
*Tricellula inaequalis*.

Detached conidia. From oatmeal agar culture, mounted in water.

or produce several more series of cells thus resulting in a compound conidiophore (Fig. 13), not unlike the conidiophores which develop in the pionnotes of a *Fusarium*. The conidium measuring  $10-12 \times 5-6.5 \mu$  consists of three cells, the basal cell  $4.5-6 (5) \times 2 \mu$  is oval, the second cell  $5-6 (5.5) \times 2 \mu$  growing out from its apex and remaining in line with the first, is also oval and slightly tapering. From near the top of the basal cell, but somewhat to one side, the third cell grows out. This lateral cell,  $4.5-6 (5) \times 2 \mu$ , always differing in shape from the other two, is boat shaped. It is attached to the basal cell with its convex side, generally in such a way that the bigger portion of the boat points in the same direction as the basal cell. The three cells are connected by

temperature was below  $2^{\circ}\text{C}$ ., the sky was clear, no wind, low humidity. During the period when it was found the fungus counts were low, with yeasts predominating."

The mould is characterized by three-celled hyaline spores (phialospores), liberated in slime (MASON, 1937). They develop on more or less compound conidiophores. The conidiophore arises from the hypha as a small bud, having attained a length of  $4-7 \mu$ , one to several buds grow out from its tip; these either develop directly into spores (simple conidiophore, Fig. 15)

Fig. 15. *Tricellula inaequalis*.

Development of a simple conidiophore in a hanging-drop culture. The conidium A was shed between 11.30 a.m. and 12.10 p.m., it remained for some time on top of the conidiophore, out of focus. Conidium B was liberated and turned over between 12.10 and 2.0 p.m. Conidium C which began to develop its second cell towards 10.0 a.m. was liberated at 3.0 p.m.

narrow constrictions (Fig. 14). It is rather difficult to ascertain whether this connection is an isthmus or whether septa develop between the cells. In mature spores, stained with cotton blue or iodine, no protoplasm could be detected in the very narrow constrictions, so probably the cells of the conidium are separated by a cell wall. The three cells, however, remain firmly together, even when roughly handled. There is not much difference between the phialides and the two straight cells of the spore, only the growth of the lateral cell shows that the cell pair is going to develop into a conidium. Observations on a hanging-drop culture (cherry decoction) show that the spore, just as the conidiophore, develops by producing

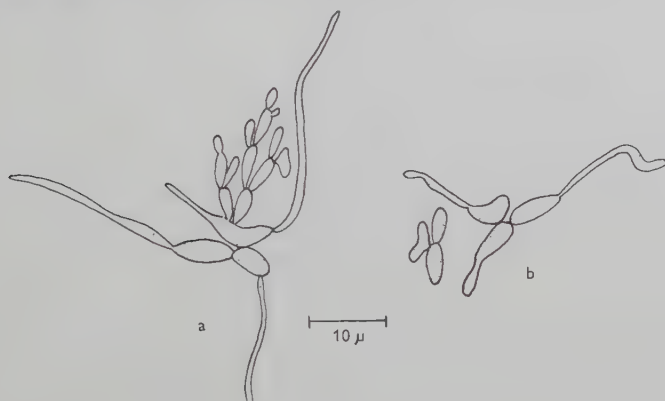


Fig. 16. *Tricellula inaequalis*.

Conidia germinated in a drop of cherry decoction, mounted in lactic acid with cotton blue.

*a.* Germinated conidium producing conidiophores.

*b.* Swollen conidium producing germ tubes; a conidium, not germinated, from the same mount drawn for comparison in size.

buds, not unlike buds from yeast cells (Fig. 15). When the second cell of the spore has reached about half its definite size, the bud of the lateral cell begins to grow out from near the tip of the basal cell (Fig. 15, development of conidium *c*). The mature spore is easily liberated from the phialide. In less favourable culture media (malt agar) a number of spores already get detached at the two-cell stage. Sporulation may occur very early; germinating spores were seen to produce conidiophores in addition to germ tubes (Fig. 16*a*).

**Cultures:** in a drop of water, malt extract or cherry decoction,

the spores germinate readily, sending out germ tubes from the free end of each cell. When germinating, the cells swell to almost double their size (Fig. 16*b*). On malt, cherry and oatmeal agar plate cultures, at room temperature, the fungus reaches a diameter of 35 mm in 4 weeks. On these media hardly any aerial mycelium develops, only a few hyphal fascicles grow on the transfer. The growth is glabrous and the surface slimy where it is richly sporulating. On malt agar the culture is raised in the centre developing many fine cerebriform wrinkles, with radial folds towards the margin, sporulation poor. In hanging-drop cultures of malt extract conidiophores grow out, but spore development is very poor. On cherry decoction agar the culture is somewhat folded in the centre, with a broad, slightly zonate, flat margin, sporulation good. Abundant sporulation occurs on starchy media such as oatmeal and cornmeal agar. On oatmeal agar the growth is flat, without folds or wrinkles, a few hyphal fascicles may occur on the transfer. The colour of the culture is "light pinkish cinnamon" (RIDGWAY, 1912). The surface is covered with a slimy spore layer up till the outer 5 mm margin which shows an almost colourless "appressed" (LONG and HARSCH, 1918) growth.

No description of this curious fungus could be found, so it seems to be a new species. Because of the three armed slime spores the species can best be ranged with the subfamily *Staurosporae* of the *Gloiosporae*, following the classification of the Hyphomycetes proposed by WAKEFIELD and BISBY (1941). In SACCARDO's system this fungus would have been ranged with the *Staurosporae* of the *Tuberculariaceae*. FRESENIUS (1852) described and figured a fungus, *Triglyphium album*, with three armed hyaline spores developing in a greyish slime. In the diagnosis of his genus, however, he describes the branches as arising from the middle of the cylindrical portion of the spore, and thinner than this — "sporae tridentis eseptatae, ramis e medio sporae exsertis tenuioribus" —. This spore formation is too different from the spore development as observed in the fungus under consideration, to allow an emendation of FRESENIUS' genus in such a way that it could contain this species. It is therefore proposed to make the present fungus the type of a new genus. Because of the spore shape the name *Tricellula inaequalis* is suggested. The natural habitat of this fungus is unknown, as it was collected from the air. This slime spore must have been dispersed by wind, but this is not unusual as e.g. macroconidia of *Fusaria* have also been reported from spore counts from the air.



*Tricellula* n.gen.

Fungus with branched, septate mycelium. Conidiophore simple or compound. Conidium (phialospore, gloiospore) three celled. Two cells in line with each other, third cell attached sidewise to the basal cell of the spore. The three cells are connected by narrow constrictions.

*Tricellula inaequalis* n.sp.

Fungus with branched, septate, hyaline mycelium. Conidiophore simple or compound, producing groups of phialides. Conidium  $10.5 \times 5.5 \mu$  three celled, each cell measuring  $4.5-6 \times 2 \mu$ . Two cells oval, in line with each other, the second budding out from the tip of the first. The third cell, boat shaped, grows out from near the apex of the first, slightly to one side. The three cells connected by narrow constrictions. Spores liberated in slime.

Natural habitat unknown, collected from the air. Bergen, Norway.

*Tricellula* gen.nov.

Mycelium ramosum, septatum. Conidiophora simplicia aut composita. Conidia (phialosporae, gloiosporae) singula constantia e ternis cellulis. Duo cellulae eodem versae, cellula tertia oblique haeret cellulae basali conidii. Tres cellulae iunctae sunt artis iuncturis.

*Tricellula inaequalis* sp.nov.

Mycelium ramosum, septatum, hyalinum. Conidiophora simplicia aut composita, formantia phialidas conglobatas. Conidia  $10.5 \times 5.5 \mu$ , singula e ternis cellulis constantia; unaquaeque cellula  $4.5-6 \times 2 \mu$ . Duo cellulae ovatae, eodem versae, altera ex apice alterius orta. Cellula tertia cymbiformis oritur loco proximo apici primae cellulae, paulum oblique. Tres cellulae iunctae sunt artis iuncturis. Conidia liberata in limo.

Hab. ignota; collecta ex aëre. Bergen, Norvegia.

## References.

- VAN BEVERWIJK, A. L. 1953. Trans. Brit. mycol. Soc. **36**, 111.  
DAVIDSON, A. M. and GREGORY, P. H. 1938. Trans. Brit. mycol. Soc. **21**, 98.  
ELLIS, M. B. 1950. Trans. Brit. mycol. Soc. **33**, 22.  
FRESENIUS, G. 1852. Beiträge zur Mykologie **2**, 44.  
HOTSON, J. W. 1912. Amer. Acad. of Arts and Sci. **48**, 225.  
HOTSON, J. W. 1917. Bot. Gaz. **44**, 265.

- LINDER, D. H. 1929. Ann. Mo. bot. Gdn. **16**, 227.
- LONG, W. H. and HARSCH, R. M. 1918. J. agric. Res. **12**, 33.
- MASON, E. W. 1937. Annotated account of fungi received at the Imperial mycological Institute List II, fasc. 3.
- RIDGWAY, R. 1912. Color standards and Color Nomenclature. Washington D. C.
- SCHULZER, S. 1877. Flora **60**, 270.
- WAKEFIELD, E. M. and BISBY, G. R. 1941. Trans. Brit. mycol. Soc. **25**, 49.
-

(From the National Institute for Public Health, Utrecht, Holland).

## THE FLOCCULATION OF TETANUS TOXIN AND TOXOID. I.

by

**J. D. VAN RAMSHORST** and **H. RIJKS**

(Received May 26, 1953).

For the determination of the antitoxin-binding power of diphtheria toxin and toxoid the flocculation method described by RAMON and DESCOMBEY (1926) has been used for some decades now. This method is based on the fact that, when mixtures of a constant quantity of toxin (or toxoid) and increasing quantities of antitoxin (serum) are prepared in a number of tubes, although flocculation eventually occurs in all the tubes, it sets in first in the tube in which the components just neutralize one another. This so-called initial flocculation, therefore, indicates the point of equivalence. The titre of the toxin or toxoid, expressed in flocculation units per ml (Lf/ml), being known, one can therefore calculate the titre of the antitoxic serum which is used, expressed in Antitoxin Units per ml (A.U./ml), and vice versa. This method gives practically no trouble.

When, however, one tries to determine the antitoxin-binding power of tetanus toxin or toxoid in this way, things are quite different. True also in this case a flocculation is seen to set in at the point of equivalence, as has already been pointed out by RAMON and DESCOMBEY (1926), but if the series of toxin-antitoxin mixtures is only made long enough, one nearly always finds several flocculation zones, so that the results are no longer unambiguous.



The flocculation picture which we get depends on the origin both of the antitoxic serum and of the toxin or toxoid. In 1937 RAMON reported that he had at his disposal a special serum, which caused only one flocculation, namely at the point of equivalence. This serum he had obtained by immunizing horses in a very special way. Several other publications on this problem have come out. On the whole, serums purified by pepsin digestion proved to be more suitable than crude serums, but even with the former one often gets several flocculations, as was pointed out by MOLONEY and HENNESSY (1944) and others. These workers also developed some methods to tell the "true" flocculation from "false" ones.

The origin of the toxins or toxoids is also important. The experiments described below were made with toxins and toxoids that have been obtained from the medium used at the National Institute for Public Health for routine production after 10-days' incubation. This is a modified Tarozzi broth, which is still prepared in about the same manner as described by PONDMAN (1939). This broth is of the following composition: 500 g veal, 10 g peptone Witte, 5 g NaCl, 1000 ml water, 1.2% glucose. Final pH 5.8—6. The meat is added in the form of cubes of about 1 cm<sup>3</sup>. Nowadays, however, the flasks are simply plugged with cotton-wool without using paraffin or other precautions to ensure an anaerobic condition. The broth with the meat is sterilized in portions of 700 ml in litre roundbottles and then inoculated with 1 ml of an inoculated and incubated seeding-culture, which has been produced in a broth prepared in the same way.

Toxins prepared from different batches of this broth all behaved in essentially the same way with regard to flocculation etc, though, naturally, they proved to differ in potency. The same applies to the toxoids prepared from these toxins. However, it would be wrong to take it for granted that conclusions based on these data are also applicable to toxins and toxoids of different origin. We hope to refer to this later.

The flocculations were all carried out with purified Wellcome tetanus serum (pepsin method), batch R.D. 948 A. A serum from the Statens Serum Institut at Copenhagen, purified by the HANSEN method (1948), proved to behave in exactly the same way, as regards flocculations.

## FLOCCULATION.

The principle of the flocculation reaction has already been stated. The practical application was as follows: 4 ml toxin or toxoid was put into a series of tubes, after which increasing amounts of serum were added. The differences between these amounts were rather small, usually 2—5 %, a little larger when Lf-values were low. The tubes were incubated at 45°C. and closely watched all the time. This is necessary, as the flocculations may spread fairly rapidly, especially when the increases have been relatively small. The tubes that we used were cleaned very carefully by chromic acid treatment followed by steaming. Otherwise disturbing irregularities may occur. Before shaking the toxin-serum-mixtures, we covered the tubes with a piece of clean stanniol, so that the fluid did not come into contact with the fingers. In this way we tried to determine the Lf of a number of toxins and toxoids <sup>1)</sup>.

Each consecutive tube of a certain series of tubes contains a somewhat larger quantity of serum (antitoxin), expressed in anti-toxin units (A.U.), than the preceding, e.g.:

|          |    |    |    |     |     |     |     |     |     |     |     |     |     |     |     |     |    |
|----------|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
| Tube no: | 1, | 2, | 3, | 4,  | 5,  | 6,  | 7,  | 8,  | 9,  | 10, | 11, | 12, | 13, | 14, | 15, | 16  |    |
| A.U.     | :  | 4, | 8, | 12, | 16, | 20, | 24, | 28, | 32, | 36, | 40, | 44, | 48, | 52, | 56, | 60, | 64 |

every tube containing 4 ml of toxin or toxoid. If we see flocculation set in in the fifth tube (and assuming that this is the "true" flocculation), this means that 4 ml of toxin is neutralized by 20 A.U., i.e. the toxin contains  $20/4 = 5$  Lf/ml. If this is indeed the only flocculation, everything is simple. But this never appeared to be the case, for at about the same time as the first flocculation, or sometimes a little later, a tube further down begins to flocculate, suppose, for instance, tube no. 15 in our example. This would therefore mean: 4 ml toxin equals 60 A.U., or: the toxin contains  $60/4 = 15$  Lf/ml. The flocculations develop in both directions till sooner or later it becomes impossible to tell them apart.

Of course, only one of these flocculations can indicate the true point of equivalence of toxin and antitoxin, and consequently only one of the flocculation values thus obtained can be properly referred to as Lf. However, so long as we do not know which of these flocculations is the right one, the only thing we can do is to apply the

---

<sup>1)</sup> In this article Lf and A.U. always refer to the new international units.

term "Lf" to all of them. Nevertheless, we must not lose sight of the fact that any given toxin or toxoid can have only one correct Lf.

TABLE 1.

| Toxin or toxoid          | „Lf” |    |    |
|--------------------------|------|----|----|
| Toxoid mixture I (crude) | 5    | 20 | 64 |
| „ I (ultr.)              | 5    | 19 | 61 |
| „ II (crude)             | 4    | 30 | 88 |
| „ II (ultr.)             | 3½   | 22 | 86 |
| Toxin 626 (crude)        | 9    | 40 | 79 |
| „ „ (ultr.)              | 3    | 36 | 70 |
| „ 628 (crude)            | 5½   | 45 | 84 |
| „ „ (ultr.)              | 5½   | 45 | 80 |
| „ 633 (crude)            | 7    | 42 | 88 |

Kf for the lowest Lf 2 à 3 hours, for the second 1 à 2 hours, for the highest ¾ à 1 hour (undiluted).

Table 1 gives the flocculation values thus found for a number of toxins and toxoids, both for crude and for ultrafiltered products (for the ultrafiltration method, see TASMAN and VAN RAMSHORST (1952)).

As this table shows, three flocculations were usually observed. The ultrafiltered toxins and toxoids were diluted to 90%—100% of the original quantity. All the components that caused the three flocculations in the "crude" toxin or toxoid were retained in the ultrafiltration process, usually even up to 80%—100%.

Generally the flocculations are distinct and sufficiently far apart to make a sharp distinction possible. If the series is started with increases of about 10 %, the impression is gained that the flocculations really emanate from one point, *viz.*, the classic "initial flocculation". But if the increases are smaller, "initial zone" is a better term with reference to the higher flocculations.



## THE CORRECT POINT OF EQUIVALENCE.

The first question rising is: does any of these flocculations actually agree with the true point of equivalence, i.e. the point at which toxin (or toxoid) and antitoxin exactly neutralize each other, so that after the flocculation neither of the components is present in its free state? If so, which is this flocculation? This was ascertained both for toxin and for toxoid by means of animal tests.

a. Toxoid determination<sup>1)</sup>.

It can be ascertained by means of an animal test how much of an unknown toxoid is bound by a certain quantity of antitoxic serum of known strength. Thus an *in vivo* measure for computing the antitoxin binding power of the toxoid in question is available. This method can be summarized as follows (see fig. 1 on the left).

Mixtures of a constant quantity of serum and gradually increasing quantities of the unknown toxoid are kept at room temperature for 24 hours. A certain quantity of toxin is then added to each mixture. After 1 hour 0.5 ml from each tube is injected intravenously into mice. The quantity of toxin is rather large, but relatively small with respect to the quantities of toxoid and serum. If in a certain mixture of serum and toxoid the

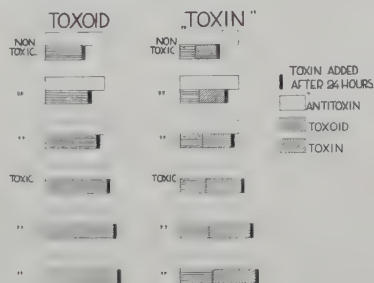


Fig. 1.

latter is in excess, the test-dose of toxin is not neutralized and the mice injected with the mixture develop tetanus symptoms or die. If, however, the serum is in excess, the mice survive.

Whenever possible, the toxoid was diluted to about 15—30 units per ml. Increasing quantities of this dilution were then added to a constant quantity of serum (12 A.U.). After 24 hours the test-dose of toxin was then added. The volume was kept constant by the addition of saline containing  $\frac{1}{2}\%$  peptone. The following is a detailed example of this technique:

<sup>1)</sup> The method here described is based on private information received from Dr I. SCHEIBEL, Statens Serum Institut, Copenhagen.

TABLE 2.

|   | toxoid<br>in ml | serum<br>in ml<br>(55 A.U./ml) | toxin solution<br>in ml<br>(test-dose) | saline + $\frac{1}{2}$ %<br>peptone<br>in ml |
|---|-----------------|--------------------------------|----------------------------------------|----------------------------------------------|
| a | 0.30            | 0.22                           | 1.20                                   | 1.28                                         |
| b | 0.38            | "                              | "                                      | 1.20                                         |
| c | 0.48            | "                              | "                                      | 1.10                                         |
| d | 0.60            | "                              | "                                      | 0.98                                         |
| e | 0.76            | "                              | "                                      | 0.82                                         |
| f | 0.90            | "                              | "                                      | 0.68                                         |
| g | 1.20            | "                              | "                                      | 0.38                                         |

The toxin solution contained 1 mg of powderized toxin per ml and the dose was chosen in such a way that it was neutralized by about 0.25 A.U.

If in the above example the first three mixtures (a, b and c) were atoxic and d, e, f and g proved toxic, this means that 0.22 ml serum of 55 A.U./ml ( $= 12$  A.U.) can completely bind 0.48 ml toxoid, but not 0.60 ml toxoid. So 12 A.U. bind about 0.5 ml toxoid, i.e. the toxoid has an antitoxin-binding value of  $12 \cdot 0.5 = 24$  (henceforth to be expressed as A.B.V.  $= 24$ ).

Of course, the above scheme can only be used for toxoids having an A.B.V. of about 15 or more. But, within certain limits, the technique used is also applicable to toxoids that have a lower A.B.V. In that case the quantity of serum and the test-dose of toxin has only to be reduced in the same proportion.

#### b. Antitoxin-binding power of toxin in vivo.

The method just described can also be used to determine the antitoxin-binding power (A.B.V.) of a "toxin". Tetanus toxin may pass into tetanus toxoid in various ways. When toxoid is prepared for active immunization against tetanus, this transition is effected by formol. However, there may also be a more or less "spontaneous" transition, which probably takes place already during the growth of the tetanus culture. (This need not necessarily mean that this "spontaneous" toxoid is identical with "formoltoxoid"! ). Crude tetanus "toxin" (and purified "toxin" too) may therefore contain a certain quantity of toxoid.

In this case the total antitoxin-binding power (A.B.V.) of these two components is determined together (see fig. 1, on the right).

c. The toxicity of fresh "toxin"-antitoxin mixtures.

When mixtures of increasing quantities of antitoxic serum and a constant quantity of "toxin" (i.e. toxin proper + toxoid, if any) are injected into mice, some of these mixtures (those with a lot of serum) will be atoxic, the others toxic.

From the limit thus found the amount of antitoxin required to deprive a given "toxin" of its toxicity can be deduced.

If the "toxin" really contained nothing but toxin proper, one would expect the quantity thus found to agree with A.B.V. (total toxin + toxoid, if present) determined by method b. For in this total the quantity of toxoid will then be = 0. If a lower value is found, this would indicate a certain percentage of toxoid in the "toxin".

However, the absolute value of this percentage is certainly not to be deduced from this. This would only be the case, assuming that first all the toxin proper is bound by the antitoxin, and then the toxoid which is also present. It is not unlikely that the toxin proper is bound more quickly (for a toxin also flocculates more quickly than a toxoid), but some of the toxoid will probably be bound simultaneously with the toxin proper.

This means that the figure thus found is a measure of the maximum quantity of toxin proper that can be present. Therefore we thought it right to call this the maximum toxin value, abbreviated M.T.V. The possibility of a different interpretation of the results of this experiment will be discussed below.

Especially the methods mentioned under a and b were used for the further determination of the relation between flocculation-values and point of equivalence.

FLOCCULATION-VALUE AND A.B.V. OF TOXOIDS.

An ultrafiltered toxoid mixture (toxoid I) showed three flocculations: **125**, **475** and **1525** "Lf"/ml. Consequently, it still remained to be seen which of these 3 values represented the "true" Lf. By means of the in vivo method (a) the A.B.V. of this toxoid was found to be **115**. Allowing a margin of error, this value agrees with the lowest of the three flocculation values.

To make sure, a quantity of serum, equivalent to the lowest flocculation value considered as the "true" Lf, was added to our toxoid, the toxoid having been diluted to 25 Lf/ml (based on the lowest Lf). The mixture was kept at 45°C. until flocculation set in, and for another hour after this. The floccules were removed by centrifuging, after which flocculation values and A.B.V. were once more determined. In the dilution used in our experiment the starting material yielded three flocculation values: **25**, **95** and **305** "Lf"/ml; the centrifuged fluid only two, i.e. **98** and **305** "Lf"/ml. So the lowest flocculation had now disappeared, the other two being retained. No antitoxin binding was found in the animal test either (A.B.V. = 0). In addition a control experiment was made, in which toxoid in the same dilution but without serum was kept at 45°C. in exactly the same way and then centrifuged. In this experiment the three flocculation values were found to remain unchanged.

Consequently, it is the specific toxoid that produces the lowest of the flocculations. It is removable by flocculation, the higher flocculation values remaining unaffected. These are therefore produced by other components of the crude or ultrafiltered toxoid. By ultrafiltration one chiefly obtains a concentration, while some non-protein products are removed at the same time. Nearly all the proteins are left in the residue and can only be separated by another method, to be described later.

#### FLOCCULATION-VALUE AND A.B.V. OF TOXINS.

An ultrafiltered tetanus toxin (toxin 628) was found to have three flocculation values, *viz.*, **128**, **1046** and **1933** Lf/ml. After dilution to 20 Lf/ml, calculated for the lowest Lf value (128), A.B.V. was determined by means of the animal test described above sub b. A.B.V. was found to be 16—18, which tallies well with the lowest flocculation value.

At the same time mixtures of 4 ml diluted toxin (diluted to 10 Lf/ml, lowest flocculation) and increasing quantities of serum (200 A.U./ml) were prepared in exactly the same way as for the Ramon titration. These mixtures were now injected into mice in four different ways: 1. immediately after mixing, 0.5 ml subcutaneously; 2. ditto, 0.5 ml intravenously; 3. after one hour's standing at room temperature, 0.5 ml subcutaneously; 4. ditto, 0.5 ml intravenously. In each of these four cases results were identical. Quantities of serum calculated for 1 Lf and 3 Lf were insufficient



to neutralize the toxin (and consequently the mice died), whereas larger quantities of serum (starting from those calculated for 5 Lf) did neutralize the toxin. So evidently the neutralization of the 10 flocculation units ( $\approx 8-9$  A.B.V.) requires no more than about 4 A.U. serum! In other words the "maximum toxin value" (M.T.V.)  $= 4$ .

Similar experiments were made with fresh crude toxins. The fresh toxin No. 630 produced three flocculations: 8, 49 and 88 Lf/ml. The animal tests gave the following figures: A.B.V.  $= 7\frac{1}{2}$ , M.T.V.  $= 3\frac{1}{2}$ . So here, too, lowest Lf and A.B.V. prove to correspond well, M.T.V. being about one half of these.

As a control, the substance causing the lowest flocculation was removed from the ultrafiltered toxin No. 626 by the addition of an equivalent quantity of serum, just as described above for toxoid. Here, too, the supernatant of the floccules only gave the two higher flocculation values and the loss of the lowest flocculation proved to be coupled with the loss of its toxicity. This was shown by m.l.d. determinations (m.l.d. is the quantity of toxin that under certain standardized conditions kills a mouse in 4 days. We shall revert to this later). The original toxin had a m.l.d.  $= 1:200,000$ , the supernatant was virtually atoxic (m.l.d.  $> 1:1000$ ). In view of possible instability of the toxin when placed at  $45^{\circ}\text{C}$ . and centrifuged, a quantity of the same toxin without the addition of serum was subjected to the same treatment, but proved to have retained its original m.l.d.

**C o n c l u s i o n:** Both for toxins and for toxoids (whether ultrafiltered or not), the A.B.V., as determined by means of animal tests, appears to correspond to the lowest flocculation value. The latter is, therefore, specific for the toxin or toxoid content or the sum of these two. Considerably less antitoxin, however, is required to neutralize the toxic effect of a "toxin". This might indicate that in fresh toxin there is already a fairly large percentage of toxoid.

#### FLOCCULATION VALUES, A.B.V., M.T.V. AND M.L.D. IN THE COURSE OF THE INCUBATION PROCESS.

In order to ascertain to what extent the values mentioned change during the growing process of the cultures, 24 flasks (1 Litre capacity), containing 700 ml of the veal broth described above, were inoculated with 1 ml of a seeding-culture, in the same broth, of strain "Wellcome C.N. 655". Every day two of these flasks were

harvested in precisely the same manner. The fluid was filtered through a double thickness of paper. The first 100 ml having been thrown away, exactly 1 Litre was passed through the filter. This litre was then passed through an E.K. filter, the first 200 ml being discarded.

In some previous experiments we had already found that this method led to reliable results, as the differences between the flasks of one batch were relatively slight.

The m.l.d. was determined immediately after harvesting. This was done away from daylight. The dilutions were made with saline containing 1 % peptone. Mice of about 15 gram were injected subcutaneously with 1 ml, and the quantity that kills mice on the 4th day after injection was taken as m.l.d.

Table 3 and fig. 2 show how the flocculation values change during the incubation of the cultures. Especially during the first few days we found some flocculations that did not quite fit into the picture. In this early stage the flocculations are not very distinct and the flocculation times (Kf) are very long, which renders observation

very difficult. Apart from these small deviations, the flocculation values can be grouped into three clear curves.

The T curve is the toxin curve. The A curve (highest flocculation value) shows a sudden drop after 9 or 10 days. Then there is a B curve and probably a C curve. The latter was more distinct in another analogous experiment than in the one summarized in fig. 2: The flocculations forming the C curve, however, are so very close to those of the B curve, as

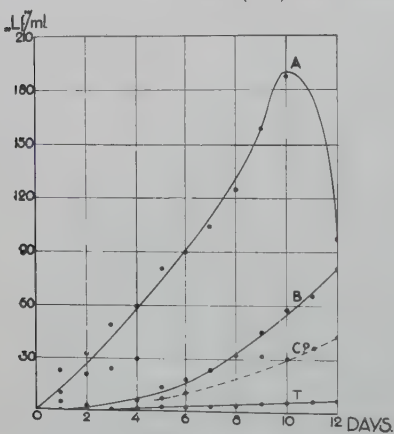


Fig. 2.

some times to be indistinguishable.

In Table 4 are the lowest flocculation value, A.B.V., M.T.V. and m.l.d. recorded. It will be seen that:

1. The lowest flocculation value closely parallels the A.B.V. as determined by the mouse test. This confirms the identity of these two values.

2. The toxicity, as given by m.l.d. determination, naturally in-

TABLE 3.

| Days after inoculation | Lf and Kf (hours) |               |                |                            |
|------------------------|-------------------|---------------|----------------|----------------------------|
| 1                      | < 1 ( $\pm$ 19)   | 5 ( $\pm$ 19) | 10 ( $\pm$ 19) | 24 ( $\pm$ 19)             |
| 2                      | 3? (18)           | 20 (12)       | 25 (8)         | 34 (8)                     |
| 3                      | 1 (9.15)          | 5 (9.15)      | 25 (7.30)      | 53 (3.45)                  |
| 4                      | 1 (6)             | 7 (6)         | 30 (5.10)      | 59 (3.15)                  |
| 5                      | 2 (9)             | 8½ (5)        | 15 (5)         | 86 (2.30)                  |
| 6                      | 2 (4.30)          | 11 (2.45)     | 19 (2.40)      | 90 (2)                     |
| 7                      | 3 (4.30)          | ?             | 26 (1.55)      | 106 (1.50)                 |
| 8                      | 4 (2)             | ?             | 34 (1)         | > 124 (1.30)               |
| 9                      | 4½ (2.15)         | 34 (1.15)     | 48 (1.15)      | 168 (2.15 <sup>1</sup> )   |
| 10                     | 5 (2.15)          | 34 (1)        | 62 (1)         | > 200 (2.30 <sup>1</sup> ) |
| 11                     | 6 (2.75)          | 40 (1)        | 70 (1)         | 186 (2.45 <sup>1</sup> )   |
| 12                     | 7 (2)             | 46 (0.45)     | 88 (0.45)      | 104 (2 <sup>1</sup> )      |

<sup>1</sup>) flocculated in dilution 1 + 1.

creases during incubation and still does so on the 12th day. The toxicity might be expected to run parallel to Lf, in other words, the ratio m.l.d./Lf remain approximately constant. But this does appear to by not means the case (also see fig. 3). This ratio very

TABLE 4.

| Days after inoculation | Lowest flocculation value Lf/ml | A.B.V. (toxin + toxoid mouse test) | "M.T.V." ("max. toxin proper") | m.l.d. (1% pep-tone) | m.l.d./Lf | "M.T.V." in % of A.B.V. |
|------------------------|---------------------------------|------------------------------------|--------------------------------|----------------------|-----------|-------------------------|
| 1                      | < 1                             | —                                  | —                              | —                    | —         | —                       |
| 2                      | ≤ 1                             | —                                  | 0.05                           | 1/900                | —         | —                       |
| 3                      | ≤ 1                             | 0.9                                | 0.4                            | 1/3000               | 3.000     | 44                      |
| 4                      | 1                               | 1.0                                | 0.5                            | 1/9000               | 9.000     | 50                      |
| 5                      | 2                               | 1.4                                | 0.9                            | 1/25000              | 12.000    | 64                      |
| 6                      | 2                               | 2.0                                | 0.8                            | 1/20000              | 10.000    | 40                      |
| 7                      | 3                               | 2.7                                | 1.1                            | 1/40000              | 13.000    | 41                      |
| 8                      | 4                               | 4.3                                | 1.5                            | 1/90000              | 22.000    | 35                      |
| 9                      | 4½                              | 4.4                                | 2.0                            | 1/150000             | 33.000    | 46                      |
| 10                     | 5                               | 4.4                                | 2.1                            | 1/180000             | 36.000    | 48                      |
| 11                     | 6                               | 5.9                                | 2.5                            | 1/350000             | 70.000    | 42                      |
| 12                     | 7                               | 10                                 | 4.5                            | 1/700000             | 100.000   | 45                      |

markedly increases during incubation and for one single culture one can — within certain limits — find any m.l.d./Lf ratio, quite according to the moment of harvesting.

3. Here, too, as in the case of the toxins already discussed, the quantity of antitoxin required to neutralize the toxic effect proved to be only half as large as the quantity that is harvested or floccu-

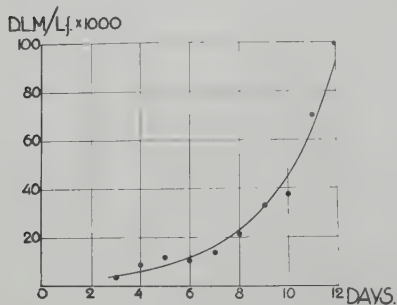


Fig. 3.

lated, that is to say M.T.V. is only the half of Lf or A.B.V. In the right hand column this is expressed in percentages. Assuming that the quantity thus found is indeed a measure of the maximum percentage of toxin proper, this would mean that from the very beginning the culture contains at least as much toxoid as toxin.

#### THE OTHER FLOCCULATIONS.

It is a well-known fact, that, besides the toxin proper or s p a s m i n, crude tetanus toxin also contains other components, the most important being t e t a n o l y s i n, which has a haemolytic effect. In the disease this haemolytic effect is probably of little or no importance, as even small concentrations of normal serum prevent haemolysis (KERRIN, 1930). It is also known, that cultures of *Cl. tetani* liquify gelatin (p r o t e o l y t i c e f f e c t). The toxin also has this effect, particularly when concentrated by ultra-filtration. The addition of such a concentrated toxin to a gelatin solution can prevent gelatinisation.

Whether these facts are in any way connected with the flocculations is as yet unknown. To find this out, fractionation of the flocculating components is essential. There is scope for further investigations in this field. So far, attention has been mainly concentrated on the isolation of the toxin or toxoid proper for routine production of the prophylactic (TASMAN and VAN RAMSHORST, 1952).

When subjected to fractionated salting-out with ammonium sulphate, the toxoid proper is found to be precipitated first, usually by 15—30% (Fig. 4, curve T). The component causing the highest flocculation is precipitated by much higher concentrations (40—70%,



curve H). This fraction is completely removed by the routine purification of toxoid. When injected into guinea pigs, this high-flocculating fraction proved to cause no measurable antitoxin production, the animals thus treated having no protection against 10 m.l.d. tetanus toxin.

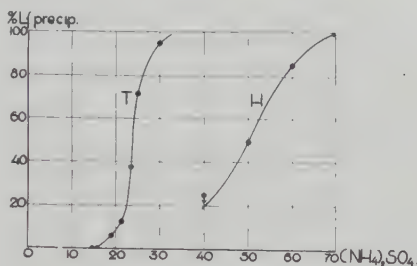


Fig. 4.

The salting-out curves of the toxoid components which cause the intermediate flocculations have not yet been sufficiently investigated.

#### THE RATIO m.l.d./Lf.

The ratio m.l.d./Lf is a concept that is frequently used in the existing literature on tetanus toxin. Sometimes this ratio is regarded as a measure of the toxoid content of the "toxin" (EISLER, 1952). There may be some truth in this idea, but it is certainly too simplified. As already stated, this ratio depends on the stage of growth (table 4).

Comparison of the absolute values that various authors give for the ratio m.l.d./Lf is somewhat difficult, their definition of m.l.d. is not always the same and many of them even fail to mention how their m.l.d. is determined. Besides the m.l.d. is strongly affected by various additions to the saline which is used for diluting, especially by peptone. It has been our experience that the addition of 1% peptone causes the m.l.d. to increase by a factor 3.5 or 4. This is sometimes interpreted as a stabilization of the toxin in weak dilutions by peptone. But this is hardly likely. For the effect is immediate and one always finds the same factor 3—4. If the toxin was so unstable as to be already seriously weakened by dilution with saline, one could not expect any reasonably constant results when determining m.l.d. with saline for a diluent and consequently it is difficult to explain how the "potentiation factor" of peptone can assume a constant value. Indeed, most authors take a different view and believe that the toxic effect is strengthened ("potentiation"; among others ZUGER *et al.*, 1939; FRIEDEMANN and HOLLANDER, 1943).

PILLEMER, WITTLER and GROSSBERG (1946) believe they obtained practically pure, or at least crystalline tetanus toxin. Their crude

product contained 10.000 m.l.d./Lf, their final product about 18.000 m.l.d./Lf; MOLONEY and HENNESSY (1944) always found about 10.000 m.l.d./Lf for their crude toxins. EISLER (1952) found much higher values. In his "best" toxins he found about 25.000 m.l.d./old Lf, i.e. abt. 50.000 m.l.d./Am. Lf, and he assumed that those toxins showing a lower value contained a proportional quantity of toxoid. Unfortunately, EISLER does not mention what diluent he used for his m.l.d. determination. In some cases, even, he found far higher values.

Table 4 shows that the ratio m.l.d./Lf is a function of the period of incubation; it increased right up to the 12th day and can perhaps increase still further. The maximum value found by us was about 100.000 m.l.d./Lf, but this m.l.d. had been determined in a peptone dilution and corresponds to about 25.000 m.l.d./Lf in a saline dilution (factor 3.5—4).

If, assuming that the above described max. toxin value (M.T.V.) actually expresses the maximum content of toxin proper, we calculate the ratio m.l.d./M.T.V., we find a greater value, *viz.*, 40.000—45.000 m.l.d./M.T.V.

But as long as the technique of m.l.d. determination has not been very strictly standardized, one cannot attach very much value to these absolute figures.

Meanwhile the rapid increase of the ratio m.l.d./Lf has still to be accounted for. EISLER (1952) thinks that this ratio is a measure of the toxoid content of the "toxin". If this is correct, the conclusion must be drawn that at first (when the ratio m.l.d./Lf is very small) the cultures contain hardly any toxin, but practically only toxoid. As the bacteria grow, more and more toxin is produced and relatively less toxoid. This might be explained by assuming that for some reason (concentration?) toxin changes quickly into toxoid in young cultures, but does so more slowly as the culture becomes older.

The ratio between maximum toxin value (M.T.V.) and Lf, however, remains constant during the incubation, and is always about = 1 : 2, which means, therefore, that 1 A.U. together with 1 Lf produces flocculation, but that  $\frac{1}{2}$  A.U. already suffices to detoxify the same quantity of "toxin". It is a remarkable fact that we have so far always found M.T.V./Lf to be about  $\frac{1}{2}$ . This consistency gives ample reason for doubting the existence of any relation between M.T.V. and the proportion of toxin proper. It may be

rather suggested that there is no toxoid production at all, but that the toxin molecules which are formed in the beginning may be less toxic than those formed later. Indeed, several authors (FRIEDEMANN and HOLLANDER, 1943; LLEWELLYN-SMITH, 1944) are of the opinion that the tetanus bacterium can produce multiple toxins which vary greatly as to toxicity but are indistinguishable as to their antigenic effect. It is not yet possible to solve this difficult problem.

### S u m m a r y.

1. The flocculation of tetanus toxins and toxoids produced in a modified Tarozi broth was made the subject of an exhaustive study.
2. At least three flocculation zones were found.
3. The lowest flocculation value always proved to agree with the true point of equivalence of the antitoxin-binding (A.B.V.).
4. When mixtures of toxin and antitoxin were injected into mice, only about half the total quantity of antitoxin that can be bound (as appears from A.B.V. or Lf) appeared to be required for the neutralization of the toxic effect. The quantity of toxin thus found was indicated as M.T.V. So this M.T.V. is approximately  $\frac{1}{2} \times$  A.B.V. or Lf.
5. During the incubation of tetanus cultures all flocculation values (3 or 4) increase steadily.
6. The d.l.m. also increases regularly, but much more quickly, hence the ratio m.l.d./Lf increases very considerably during incubation.
7. At the same time the ratio M.T.V./Lf remains constant, i.e. about  $\frac{1}{2}$ .
8. The possible theoretical explanation of the different problems was discussed.

### L i t e r a t u r e.

- EISLER, M. 1952. *Z.f. Hyg.* **135**, 577.  
FRIEDEMANN, U. and HOLLANDER, A. 1943. *J. Immun.* **47**, 23.  
HANSEN, A. 1948. *Acta Path.* **25**, 460.  
KERRIN, J. C. 1930. *Brit. J. Exp. Path.* **11**, 153.  
LLEWELLYN-SMITH, M. 1944. *Bull. Org. Hyg.* **10**, 138.  
MOLONEY, P. J. and HENNESSY, J. N. 1944. *J. Immun.* **48**, 345.  
PILLEMER, L., WITTLER, R. and GROSSBERG, D. B. 1946. *Science* **103**, 615.

PONDMAN, A. 1939. *Acta Leidensia* **14**, 176.

RAMON, G. 1922. *C.R. Soc. Biol.* **74**, 661, 711.

RAMON, G. 1937. *C.R. Soc. Biol.* **124**, 414.

RAMON, G. and DESCOMBEY, P. 1926. *C.R. Soc. Biol.* **95**, 434.

TASMAN, A. and VAN RAMSHORST, J. D. 1952. *Antonie van Leeuwenhoek* **18**, 357.

ZUGER, B., HOLLANDER, A. and FRIEDEMANN, U. 1939. *J. Inf. Dis.* **65**, 87.



(Microbiological Laboratory of the Zuiderzee Reclamation Works,  
Kampen, Holland).

## ON THE PRODUCTION OF LEGUME INOCULANTS IN A MIXTURE OF PEAT AND SOIL

by

**D. A. VAN SCHREVEN, D. OTZEN and D. J. LINDENBERGH**

(Received March 21, 1953).

### INTRODUCTION.

Since the first experiments with artificial cultures of rhizobia of NOBBE and HILTNER in 1890, which were not made public until February 1896, many patented culture methods for the distribution of rhizobia have been developed. Although the results obtained with these cultures have often been very disappointing and contradictory, it may be fairly said that without the use of such cultures it would have been impossible to achieve the present extensive cultivation of leguminous crops.

In the United states alone, the value of the commercial cultures distributed in 1930, amounted to \$ 1.000.000 (KNIGHT, 1930) and in 1940 and 1946 to \$ 3.000.000 and \$ 4.000.000, respectively (ERDMAN, 1948). Three types of legume inoculants are in general use: 1) cultures in moist humic material or moist peat-powder; 2) cultures on agar (jelly) and 3) liquid cultures. In the U.S.A. moist peat-powder or agar are mainly used as "carriers", but the use of the latter has been declining within recent years. According to LEONARD (1944) 11 concerns in the U.S.A. were engaged in the production of nodule bacteria cultures in 1944.

Peat-powder inoculants are usually prepared commercially by developing a heavy suspension of nodule bacteria in aerated liquid nutrients in protected tanks. When at the right stage of growth, the suspension is withdrawn and mixed thoroughly with neutralized peat-powder. Further processing may include 1) curing (allowing to stand in a bin or tub for a few days), 2) additional mixing to obtain

particle uniformity, and 3) packaging. It is then ready for the retail trade. Prepared in this manner inoculants are not pure cultures, but the presence of other organisms does not seem to affect their efficiency. Moreover contaminants are not sufficiently in evidence as a rule to affect the salability of the product (LEONARD, 1944).

The success of inoculation depends on (a) whether the proper rhizobium is absent in the soil and (b) whether the inoculum contains a sufficient number of the proper organism. Such a high percentage of success may be expected in newly reclaimed regions and in regions with many acid soils.

In the Netherlands practically all the soils which have been cultivated for a long period contain most kinds of rhizobia, but inoculation is necessary or advisable 1) on all newly reclaimed soils, 2) on soils where the legume concerned had not been grown before within the previous 4 years and 3) on markedly acid soils (GERRETSEN, 1950).

In a former article a method has been described of growing mass cultures of rhizobia in liquid medium in milk-cans (VAN SCHREVEN *et al.*, 1953). This method is only suitable, however, when it is not necessary to store the inoculant for a long time. Since September 1948 we have studied some factors influencing the growth and survival of rhizobia in mixtures of peat and soil. This was done in order to obtain inoculants containing extremely high numbers of the proper organism in a viable state, which shall in most cases be free from other microbes and can be stored for a long period.

## EXPERIMENTS.

### a. Culture experiments with soil-peat mixtures in Erlenmeyer flasks.

In September 1948 the following media were tested:

- |                                                           |                                         |
|-----------------------------------------------------------|-----------------------------------------|
| I. 45 % heavy loam                                        | II. 46 % heavy loam                     |
| 40 % alder bog peat                                       | 42 % alder bog peat                     |
| 10 % lucerne meal                                         | 10 % lucerne meal                       |
| 3 % ground wheat straw                                    |                                         |
| 1 % yeast                                                 | 1 % yeast                               |
| 0.5% mannitol                                             | 0.5% mannitol                           |
| 5 g $\text{CaCO}_3$ per 50 g of mixture                   | 5 g $\text{CaCO}_3$ per 50 g of mixture |
| III. As II but 42 % peat dust in place of alder bog peat. |                                         |

In these mixtures the quantities of heavy loam and peat had been based on dry material. The heavy loam was rich in  $\text{CaCO}_3$  and like the alder bog peat derived from the North-Eastern polder. The materials were dried and sifted beforehand. After the substrates had been mixed, they had a water holding capacity of 64.1, 61.2 and 81.6 %, respectively. The initial pH was 6.18, 6.18 and 5.55.

To three sets of 20 Erlenmeyer flasks 50 g of these mixtures were introduced and sterilized twice for 1 hour at  $125^\circ\text{C}$ . with an interval of 24 hours. The substrates were inoculated with a liquid inoculum. A sufficient quantity of this inoculum was added to these mixtures to give 50 % moisture. Immediately after the addition of the inoculum the initial numbers of bacteria were estimated by means of plate counts<sup>1)</sup>. Counts of rhizobium were made periodically in some of the flasks. Each week as much sterile water was added as had been lost by evaporation. The numbers of the rhizobia and the pH of the media are shown in table 1.

TABLE 1.

Numbers of *R. meliloti* in millions per g of wet soil-peat mixture, grown in Erlenmeyer flasks, stoppered with cotton-wool.

| After<br>....<br>days | Mixture I | pH   | Mixture II         | pH   | Mixture III        | pH   |
|-----------------------|-----------|------|--------------------|------|--------------------|------|
| 0                     | 115       | —    | 534                | —    | 807                | —    |
| 7                     | 16280     | 7.47 | 17080              | 7.41 | 13340              | 7.09 |
| 41                    | 11340     | 6.07 | 27620              | 6.06 | 9620               | 6.05 |
| 64                    | 27542     | —    | 7610               | —    | 7202               | —    |
| 192                   | 15470     | 6.80 | 5840 <sup>1)</sup> | 6.70 | 4220 <sup>1)</sup> | 6.60 |

<sup>1)</sup> Contaminated with another organism.

After one week the numbers ranged from 13000 to 17000 million per g of moist material. The best results were obtained with mixtures I and II, apparently due to the use of alder bog peat. By comparison with fresh garden soil which usually contains about 150—200 million bacteria per g (determined by means of plate counts), the numbers presented in table 1 are extremely high.

<sup>1)</sup> For all the counts, mentioned in this paper, 10 plates were used for each dilution. All media were incubated at about  $29\text{--}30^\circ\text{C}$ .

### b. Culture experiments with soil-peat mixtures in aerated milk-cans.

Alder bog peat was dried beforehand until the moisture content was 70 % and then mixed with dry heavy loam (which had been ground and sifted) and the nutrients, after which the moisture content was 48 %, somewhat more than 50 % of the water holding capacity. The milk-cans I and II were provided with 7.5 kg of this moist material. After having been sterilized twice at 125° C., with an interval of 24 hours, the milk-cans were inoculated and mixed with 200 ml of a liquid rhizobium culture (*R. meliloti*).

The composition of the mixture in the milk-cans (mixture A) was as follows:

|                    |                      |
|--------------------|----------------------|
| 44% heavy loam     | 4% CaCO <sub>3</sub> |
| 40% alder bog peat | 3% yeast extract     |
| 10% lucerne meal   | 0.5% mannitol        |

After sterilizing the medium, the pH was 7.1. The milk-cans were aerated with moist sterile air and shaken every day for half an hour. After 13 days the milk-cans I and II contained respectively 12150 and 17530 million nodule bacteria per g of moist material. The contents were put into jam jars, which were closed with a lid and placed in a refrigerator at 2° C. Fifteen days later the materials I and II contained resp. 12000 and 11050 million nodule bacteria per g. After keeping the material for 33 days at 2° C. the numbers of rhizobia were practically the same.

In another experiment the same mixture A was compared with a mixture B, containing 10 % yeast extract and with a mixture C containing 0.5 % milk-powder instead of 0.5 % mannitol.

TABLE 2.

Numbers of *R. meliloti*, grown in milk-cans for 14 days in mixtures of peat and soil in millions per g of moist material.

| Mixture |     |                |                 |                             |             | Numbers |
|---------|-----|----------------|-----------------|-----------------------------|-------------|---------|
| A,      | 3%  | yeast extract, | $\frac{1}{2}$ % | mannitol                    | } aerated   | 12390   |
| A,      | 3%  | "              | "               | $\frac{1}{2}$ % "           |             | 11950   |
| A,      | 3%  | "              | "               | $\frac{1}{2}$ % "           | } unaerated | 240     |
| A,      | 3%  | "              | "               | $\frac{1}{2}$ % "           |             | 220     |
| B,      | 10% | "              | "               | $\frac{1}{2}$ % "           | } aerated   | 15260   |
| B,      | 10% | "              | "               | $\frac{1}{2}$ % "           |             | 16930   |
| C,      | 3%  | "              | "               | $\frac{1}{2}$ % milk-powder | } "         | 12760   |
| C,      | 3%  | "              | "               | $\frac{1}{2}$ % "           |             | 12130   |



Mixture A was also used without aerating the cans. The other cans were aerated with moist air. The water contents varied between 48.2 and 52.8 %. The numbers which were found after 14 days are summarized in table 2.

A strikingly lower number of bacteria was obtained in the un-aerated cans; growth of rhizobium was stimulated more by 10 % yeast extract than by 3 % yeast extract; it makes no difference whether mannitol or milk-powder is used.

### c. Culture experiments with soil-peat mixtures in aerated milk-bottles.

300 g of the following mixtures were put into 1 litre milk-bottles.

- 1) 43% heavy loam, 40% alder bog peat, 10% lucerne meal, 3% wheat straw, 4%  $\text{CaCO}_3$ . Per kg of dry matter 100 ml yeast extract and 5 g mannitol were added.
- 2) As 1), but 20 g in place of 5 g mannitol.
- 3) As 1), but 20 g glucose per kg in place of 5 g mannitol.
- 4) As 1), but 20 g milk-powder per kg in place of 5 g mannitol.
- 5) As 1), but 30 ml in place of 100 ml yeast extract.
- 6) As 1), but with peat-detritus in place of heavy loam and alder bog peat.

After sterilization the media were inoculated with 100 ml of a culture of *R. meliloti*, which had been aerated for 2 days.

The flasks were aerated. Plate counts on mannitol agar were made after 1 and 2 weeks for which purpose the contents of 1 flask per set was examined. The results are shown in table 3.

TABLE 3.

Number of *R. meliloti* in soil-peat mixtures in millions per g of moist material grown in aerated milk-bottles.

| Mixture | After 1 week |     |            | After 2 weeks |            |
|---------|--------------|-----|------------|---------------|------------|
|         | Numbers      | pH  | Moisture % | Numbers       | Moisture % |
| 1       | 7280         | 6.9 | 51.6       | 7580          | 51.2       |
| 2       | 12150        | 7.0 | 50.2       | 11730         | 50.5       |
| 3       | 9130         | 7.1 | 50.6       | 9920          | 49.1       |
| 4       | 13660        | 6.8 | 49.4       | 13050         | 52.0       |
| 5       | 8010         | 6.9 | 50.4       | 7100          | 46.8       |
| 6       | 18900        | 6.9 | 47.5       | 14180         | 45.5       |

After one week the maximum number of bacteria per g of material was reached, as shown by the striking agreement obtained after 1

and 2 weeks between the corresponding samples. A markedly good result was obtained with peat-detritus. In this experiment 20 g mannitol gave better results than 5 g. Good results were also obtained with glucose and with milk-powder; 30 ml yeast extract had the same effect as 100 ml yeast extract.

#### d. Culture-experiments with peat-detritus mixtures in tins.

In these experiments the following mixtures were used in quadruplicate:

- 1) 83% peat-detritus, 10% lucerne meal, 3% ground wheat straw, 4%  $\text{CaCO}_3$ . Per kg of this mixture were added: 10 g yeast and 20 g milk-powder.
- 2) As 1), but without yeast.
- 3) As 1), but 73% peat-detritus and 20% lucerne meal.
- 4) As 1), but with 43% heavy loam and 40% alder bog peat in place of peat-detritus.
- 5) As 1), but with 43% heavy loam and 40% peat dust in place of peat-detritus.

Half the contents of the tins were filled with these mixtures, 800 g per tin. The mixtures had a water content of 52–55 %. The tins were sterilized twice for one hour at 125° C. with an interval of 24 hours and inoculated with a liquid culture of *R. meliloti*, strain 15. The lids of the tins were fitted with a tube, stoppered with cotton-wool. Each day the tins were shaken thoroughly. After 11 days plate counts were made of the contents of one tin of each sample; the remaining tins were tightly stoppered with a sterile rubber stopper and stored at room temperature. Plate counts of the nodule bacteria in these tins were made 55 and 139 days later. The results are given in table 4.

TABLE 4.  
Numbers of *R. meliloti* in soil-peat mixtures, grown in aerated tins in millions per g of moist material.

| Mixture | 11 days after inoculation | Stored at room temperature in closed tins for |          |
|---------|---------------------------|-----------------------------------------------|----------|
|         |                           | 55 days                                       | 139 days |
| 1       | 15620                     | 2972                                          | 1980     |
| 2       | 9720                      | 3050                                          | 4274     |
| 3       | 15080                     | 5400                                          | 3538     |
| 4       | 14440                     | 5740                                          | 990      |
| 5       | 13520                     | 2046                                          | 1304     |

The initial high numbers of bacteria decreased markedly after storing at room temperature for resp. 55 and 139 days in air-tight closed condition.

In another experiment a number of cultures of rhizobium, grown in mixtures of soil and peat were stored at room temperature, while air had free access through a wad of cotton-wool in the tube of the lid. Some data are shown in table 5.

TABLE 5.

Numbers of *R. meliloti* in soil-peat mixtures, stored at room temperature while air had free access, in millions per g.

| Mixture | Fresh inoculum | After 28 days | After 53 days | After 91 days |
|---------|----------------|---------------|---------------|---------------|
| 1       | 9620           | 8100          | 6400          | 4660          |
| 2       | 10260          | 14760         | 6740          | 6330          |
| 3       | 9080           | 11080         | 10700         | 6360          |
| 4       | 6180           | 18360         | 13360         | 4080          |
| 5       | 11140          | 12720         | 11220         | 6900          |

The data show that when air is available during storage the numbers of rhizobium decrease less rapidly than in an air-tight space. To be sure of a high number of bacteria in the inoculum at the moment of delivery, it is desirable, that a small access of air is possible. In other experiments it had already been shown, that the numbers remain high for a long period when the cultures are stored at a temperature of 2° C. It is also important to store the inoculum at 2° C. because this low temperature reduces loss of moisture.

#### e. Culture experiments with soil-peat mixtures in dispensing-bottles.

In November 1949 dispensing-bottles of 250 ml, containing 100 g of soil-peat mixture, were used. They were plugged with cotton-wool. For comparison, one litre milk-bottles were used with 250 g of the same substrate, which were aerated with an air stream. In this experiment the water content of the substrate was also varied. The mixtures were inoculated with 5 ml of a culture of *R. meliloti* (strain No. 15) and the bottles were shaken every day by hand for a short time. The composition of the medium, based on dry material, was as follows:

|                    |                      |
|--------------------|----------------------|
| 43% heavy loam     | 10% lucerne meal     |
| 43% alder bog peat | 4% CaCO <sub>3</sub> |

Plate counts were made after 9 days. The results are given in table 6.

TABLE 6.

Numbers of *R. meliloti* in millions per g of moist material, grown in dispensing-bottles stoppered with cotton-wool and in aerated milk-bottles respectively.

| Water content<br>% | Dispensing-bottles | Milk-bottles |
|--------------------|--------------------|--------------|
| 40—45              | 10420              | 13560        |
| 50—55              | 12960              | 12380        |
| 60—65              | 13120              | 15600        |
| 80—85              | 4460               | 1974         |
| 90—95              | 1348               | 1054         |
| Total              | 42408              | 44568        |

The highest numbers per g of moist material were obtained at a water content of 60—65 %. A marked decline of the numbers, however, appeared at a water content of 80—85 %. When small amounts of soil-peat mixtures were used, access of air through cotton-wool has given practically the same results as blowing air into the bottles.

In the same soil-peat mixtures the numbers of different rhizobium

TABLE 7.

Numbers of some *Rhizobium* species, grown for 10 days in dispensing-bottles, stoppered with cotton-wool.

| <i>Rhizobium</i> species              | Mean number of two bottles in millions<br>per g of moist material |
|---------------------------------------|-------------------------------------------------------------------|
| <i>R. leguminosarum</i> <sup>1)</sup> | 8070                                                              |
| " " <sup>1)</sup>                     | 6250                                                              |
| " " <sup>1)</sup>                     | 5590                                                              |
| <i>R. meliloti</i> <sup>1)</sup>      | 11750                                                             |
| " " <sup>1)</sup>                     | 12450                                                             |
| <i>R. phaseoli</i>                    | 7340                                                              |
| <i>R. trifolii</i>                    | 6460                                                              |
| <i>R. lupini</i>                      | 10130                                                             |

<sup>1)</sup> Different strains.



species were compared, by growing them in dispensing-bottles. The results are listed in table 7.

All these *Rhizobium* species showed a good growth in this medium. The highest numbers were obtained with *R. meliloti*.

In another experiment the development of *R. meliloti* was studied in the following media:

- 1) 42% heavy loam, 33% alder bog peat, 10% peat dust, 10% lucerne meal, 5%  $\text{CaCO}_3$ . To each kg, dry weight, of this mixture were added: 10 g yeast, 12.5 g glucose, 2.5 g mannitol, 0.5 g  $\text{K}_2\text{HPO}_4$  and 0.2 g  $\text{MgSO}_4$ .
- 2) As 1), but with 20% lucerne meal and without peat dust.
- 3) As 2), but without salts.
- 4) As 2), but without salts, glucose and mannitol.
- 5) As 2), but without salts, glucose, mannitol and yeast.
- 6) As 5), but with 5% peat dust and 15% lucerne meal.
- 7) As 5), but with 38% alder bog peat and 25% lucerne meal.
- 8) 75% heavy loam, 20% lucerne meal and 5%  $\text{CaCO}_3$ .
- 9) 75% alder bog peat, 20% lucerne meal and 5%  $\text{CaCO}_3$ .

In this experiment dispensing-bottles were used with a capacity of 250 ml. Each bottle was about half filled with one of these media and inoculated with 10 ml of a culture of *R. meliloti* (strain A 15). For each medium 4 bottles (a, b, c and d) were used. The moisture content was about 50 %. After incubation at 29° C. for one week, the top-end of the bottles was enveloped with cellophane paper to reduce evaporation of water and the bottles were stored at room temperature. Plate counts were made after 15 and 49 days storage. The results are given in table 8.

After 15 days high numbers of rhizobium were found in all media, especially in the first 7 media (mixtures of soil, peat and lucerne meal). In most cases the media with 20 % lucerne meal gave at first higher numbers than the first medium with 10 % lucerne meal. Medium No. 5 (with 20 % lucerne meal and without salts, glucose, mannitol and yeast) produced higher numbers after 15 days than the first medium. Mixing the heavy loam with peat was an evident improvement, since the numbers in medium 8 (without peat) were distinctly lower than those in medium 5. A mixture of neutralized alder bog peat and 20 % lucerne meal (medium 9) proved to be a better medium than a mixture of heavy loam and 20 % lucerne meal (medium 8).

After 49 days storage the numbers of rhizobium had decreased markedly. It is not clear why the numbers in the bottles 3 c and 3 d

TABLE 8.

Numbers of *R. meliloti* in different media, using dispensing-bottles which had been closed with a wad of cotton-wool (numbers in millions per g of humid material).

| Medium | Bottle | After 15 days | Bottle | After 49 days |
|--------|--------|---------------|--------|---------------|
| 1      | 1 a    | 10320         | 1 c    | 4290          |
|        | 1 b    | 12360         | 1 d    | 6350          |
| 2      | 2 a    | 16360         | 2 c    | 5950          |
|        | 2 b    | 14850         | 2 d    | 5480          |
| 3      | 3 a    | 18540         | 3 c    | 10870         |
|        | 3 b    | <sup>1)</sup> | 3 d    | 10720         |
| 4      | 4 a    | 15210         | 4 c    | 4520          |
|        | 4 b    | 12250         | 4 d    | 5240          |
| 5      | 5 a    | 14990         | 5 c    | 3990          |
|        | 5 b    | 18990         | 5 d    | 6780          |
| 6      | 6 a    | 14140         | 6 c    | 3590          |
|        | 6 b    | 4390          | 6 d    | 2640          |
| 7      | 7 a    | 16350         | 7 c    | 5870          |
|        | 7 b    | <sup>1)</sup> | 7 d    | <sup>1)</sup> |
| 8      | 8 a    | 5400          | 8 c    | 1900          |
|        | 8 b    | 6900          | 8 d    | 890           |
| 9      | 9 a    | 9720          | 9 c    | 3010          |
|        | 9 b    | 11940         | 9 d    | 2790          |

<sup>1)</sup> Unsuccessful count.

were the highest after 49 days, and were higher than e.g. in the bottles 2 c and 2 d.

To study once more the influence of lucerne meal on the development of rhizobia, the following media were tested:

- 1) The same medium as No. 1 in the former experiment.
- 2) The same medium as No. 3 in the former experiment.
- 3) As 1), but with 43 % alder bog peat and without lucerne meal.

The media were inoculated with *R. meliloti*. Counts were made in duplicate after storage at room temperature for 7, 21 and 44 days. The results are shown in table 9.

TABLE 9.

Numbers of *R. meliloti* in different media, using dispensing-bottles stoppered with cotton-wool, in millions per g of moist material.

| Medium | After 7 days | After 21 days | After 44 days |
|--------|--------------|---------------|---------------|
| 1      | 6860         | 4130          | 3800          |
|        | 6010         | 5580          | 4540          |
| 2      | 12840        | 5630          | 3050          |
|        | 13610        | 5360          | 4480          |
| 3      | 1110         | 1250          | 720           |
|        | 1260         | 1360          | 980           |

This experiment clearly demonstrates the stimulating effect of lucerne meal on the development of *R. meliloti*. This is in accordance with the work of TEMPLE (1916), who found that by the addition of lucerne meal to a soil low in fertility, the numbers of rhizobia increased markedly. The same effect may be expected in the field after ploughing under a crop of lucerne or another legume.

#### f. Effect of shaking the bottles after the inoculation.

In the former experiments the containers were shaken every day after the inoculation in order to accomplish a good mixing of the rhizobia in the media. To avoid clotting of the material, the first shaking was carried out one day after the inoculation of the medium and was always a short, gentle shaking and performed by hand. The influence of shaking was studied as follows:

- 1) The bottles were not shaken.
- 2) Shaken on the 2nd day after the inoculation.
- 3) Shaken on the 2nd and 4th day after the inoculation.
- 4) Shaken on the 2nd, 3rd and 4th day after the inoculation.

Apparently (Table 10) shaking once, on the 2nd day after the inoculation, is sufficient.

#### g. Influence of the moisture content.

The effect of the moisture content had to be studied for the medium which had proved to be the best for practical purposes, *viz.*,

|                    |                      |
|--------------------|----------------------|
| 42% heavy loam     | 20% lucerne meal     |
| 33% alder bog peat | 5% CaCO <sub>3</sub> |

TABLE 10.

Influence of shaking the media in dispensing-bottles on the number of *R. meliloti* (strain A 15) in millions per g of moist material.

| Series | Bottle | Numbers of rhizobia |
|--------|--------|---------------------|
| 1      | 1 a    | 4340                |
|        | 1 b    | 4310                |
| 2      | 2 a    | 16130               |
|        | 2 b    | 18750               |
| 3      | 3 a    | 17660               |
|        | 3 b    | 17730               |
| 4      | 4 a    | 17000               |
|        | 4 b    | 21690               |

In this experiment dispensing-bottles of 400 ml were used with 160 g of the medium. Each bottle was inoculated with 5 ml of a culture of *R. meliloti* (strain A 15). Plate counts of the bacteria in 3 bottles per sample were made after 7 days (table 11).

TABLE 11.

Influence of the moisture content on the numbers of *R. meliloti* (strain A 15). Numbers in millions per g of moist material.

| Moisture content (%) | Mean number |
|----------------------|-------------|
| 20                   | 330         |
| 30                   | 2736        |
| 40                   | 7266        |
| 45                   | 6603        |
| 50                   | 12756       |
| 55                   | 12725       |
| 60                   | 14100       |
| 65                   | 11953       |
| 70                   | 8713        |
| 80                   | 918         |

The highest numbers were found in a water content of 60 %, corresponding with 83 % of the water holding capacity.



## PROCEDURE FOR MAKING AND INOCULATING THE MEDIA.

After having been air dried the heavy loam is ground in a mill. The alder bog peat is sifted through a 2 mm sieve. As the lucerne meal is not fine enough, it is ground in a mill and passed through a 1 mm sieve. The moisture content of the mixture is brought to 60 %. When the bottles are half filled with the media, they are stoppered with cotton-wool and the top-end is wrapped with paper to prevent contamination of the cotton-wool after sterilization. The bottles are sterilized for 3 hours at 125° C. (pressure of about 20 pounds).

The media are inoculated with a liquid culture of rhizobium, which is made in the following way:

1. A diluted suspension of rhizobium is plated in 10 ml agar medium.

2. An agar slope is inoculated from one colony of rhizobium.

3. When the rhizobium has developed in the tube, a suspension of the young culture is made by adding 10 ml sterile water.

4. Three litres of a solution in a container A (fig. 1) are inoculated with this suspension. The medium is aerated with sterile air.

5. After two days this solution can be used as an inoculum for a series of dispensing-bottles. About 5 or 10 ml of the inoculum

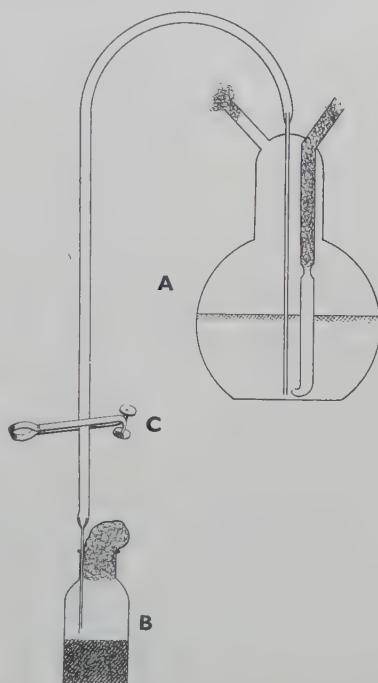


Fig. 1.

are siphoned over from the container A into the dispensing-bottle B through a rubber tube and hollow needle attached to the end. The latter is inserted between the cotton-wool plug and the wall of the neck of the dispensing-bottle after first removing the paper which covered the cotton-wool. Thus it is not necessary to remove the cotton-wool during the inoculation by which an important source of contamination is eliminated. The flow of inoculum from the receiver is regulated by means of clip C fixed on the rubber tube

and by using a stop-watch. The rubber tube and needle are sterilized with the apparatus. During the sterilization the needle was enclosed in a test tube, the mouth of which was closed with cotton-wool.

It is of great importance to use a pure inoculum. Therefore a small sample of the inoculum is siphoned over and examined by means of a phase contrast-microscope.

By using this procedure practically no contamination takes place, but as a further precaution, the inoculum of some bottles, from each set that has been inoculated with any one receiver, is examined before distribution. After the incubation for one week at 29—30° C., the top-ends of the bottles are wrapped with cellophane paper.

#### DISTRIBUTION OF THE CULTURES.

The cultures are sent immediately to "The Soil and Crop testing Laboratory" (Bedrijfslaboratorium voor Grond- en Gewasonderzoek) in Groningen. The distribution of the cultures takes place from this laboratory. In Groningen the cultures are stored at 2° C. before selling them. Samples are checked regularly by the Microbiological Department of the Agricultural Experiment Station and Institute for Soil Research T.N.O., Groningen. Results of an examination of some of these cultures, performed in 1952 by Dr HARMSSEN, are given in table 12.

In two cases contamination of the inoculum by another organism was found. The inoculum for Black medic (*M. lupulina*), which had been contaminated with a *Streptococcus* species, had been forwarded before the examination of it in our laboratory was complete, owing to the urgent need for speedy delivery. Some days after the transmission we discovered the same contamination in this inoculum. Obviously the liquid culture of *R. meliloti* which had been used for the inoculation of the bottles had been very slightly contaminated with this *Streptococcus*, for this had not been detected when the liquid culture was inspected by means of the phase contrast-microscope. As a matter of fact this inoculum would not have been forwarded, if there had been enough time for the examination. The second case of contamination, viz., that with *Bac. polymyxa*, evidently had been an accidental one, since all the five inocula for lupine of this sending, which had been tested in our laboratory, were entirely free from other microbes.

TABLE 12.

Examination of cultures in 1952 at the Agric. Exp. Sta. and  
Inst. for Soil Research, T.N.O., Groningen.

| Inoculum for         | Date | Numbers of rhizobium<br>per g of moist material | Contamination<br>noted                              |
|----------------------|------|-------------------------------------------------|-----------------------------------------------------|
| Clover               | 10—3 | $4.5 \times 10^9$                               | no                                                  |
| Lupine               | 10—3 | $18.0 \times 10^9$                              | "                                                   |
| Lucerne              | 10—3 | $20.0 \times 10^9$                              | "                                                   |
| Black medic <i>a</i> | 10—3 | $14.0 \times 10^9$                              | <i>Streptococcus spec.</i><br>$\pm 2.0 \times 10^9$ |
| " " <i>b</i>         | 10—3 | $4.5 \times 10^8$                               | no                                                  |
| Peas and Vicia       | 10—3 | $4.5 \times 10^9$                               | "                                                   |
| Clover               | 2—4  | $15.0 \times 10^9$                              | "                                                   |
| Lucerne              | 2—4  | $20.0 \times 10^9$                              | "                                                   |
| Clover               | 8—4  | $5.5 \times 10^9$                               | "                                                   |
| Black medic <i>a</i> | 8—4  | unsuccessful count                              | "                                                   |
| " " <i>b</i>         | 21—4 | $6.0 \times 10^9$                               | "                                                   |
| Lucerne              | 21—4 | $14.5 \times 10^9$                              | "                                                   |
| Black medic <i>a</i> | 21—4 | $19.5 \times 10^9$                              | "                                                   |
| " " <i>b</i>         | 21—4 | $7.5 \times 10^8$                               | "                                                   |
| Lupine               | 21—4 | $16.5 \times 10^9$                              | "                                                   |
| Clover               | 2—5  | $4.25 \times 10^9$                              | "                                                   |
| Peas and Vicia       | 2—5  | $7.5 \times 10^9$                               | "                                                   |
| Black medic <i>a</i> | 2—5  | $7.0 \times 10^9$                               | "                                                   |
| " " <i>b</i>         | 2—5  | $5.0 \times 10^8$                               | "                                                   |
| Lucerne              | 2—5  | $45.0 \times 10^9$                              | "                                                   |
| Clover               | 12—5 | $4.5 \times 10^9$                               | "                                                   |
| Lupine               | 12—5 | $13.0 \times 10^9$                              | <i>Bac. polymyxa</i><br>$\pm 3.75 \times 10^8$      |
| Black medic <i>a</i> | 12—5 | $10.25 \times 10^9$                             | no                                                  |
| " " <i>b</i>         | 12—5 | $2.5 \times 10^8$                               | "                                                   |

*a.* slow growing strain; *b.* rapid growing strain.

Moreover contamination with air-borne microorganisms, will not be harmful, if they do not seriously influence the numbers of rhizobium.

Two strains were used for the black medic inoculum (see table 12). It is interesting that the strain with a slow growth on the agar-medium has multiplied faster in the soil-peat mixture than the other strain which has a rapid growth on agar.

## COMPARISON OF OUR CULTURES WITH THOSE OF FOREIGN INOCULANTS.

TEMPLE (1916) tested a number of inoculants, made by manufacturers in the U.S.A. in 1916, but the numbers mentioned by him refer to the bacteria in the whole sample. For the cultures in which soil was used as a medium, these numbers ranged from 4840 to 17125 million (Nitragin) and from 2795 to 4354 million (The Nitra-Germ Co., Savannah, Ga) in the whole sample per acre. The highest number obtained by TEMPLE in cultures of soil and alfalfa meal was 1610 million per g. A number of commercial cultures in the U.S.A., with muck (peat) or black soil as a carrier, were tested by FELLERS (1918) *viz.*, Farmogerm, Alphano inoculant and Nitragin. The numbers of bacteria per ml, after addition of 20 ml sterile water to each sample, varied between 100.000 and 3.000.000. Many difficulties were encountered in making these counts, because the cultures were not pure and contained many species of fungi, actinomycetes and foreign bacteria. According to FELLERS few cultures containing less than 1.000.000 cells per ml were effective. The total number of bacteria in samples calculated per acre proved to be a better means of comparison. This number ranged from 600 to 36.000 million for the tested cultures. HOFER and CONN (1931) tested a number of inoculants, belonging to 8 brands, in 1931. The highest number of root nodule bacteria, out of 54 inoculants in the U.S.A. and expressed per pound of seed, was 940 million. They did not mention, however, which carriers were used in these cultures.

The highest number of rhizobium found by HOFER (1938) in inoculants with sand as a carrier was 166 million per pound of seed (clover). Cultures that carried fewer than 1.000.000 legume bacteria per pound of seed usually failed to produce nodules in the greenhouse; those which carried 8.000.000 or more formed nodules upon their host plants. Cultures with counts between these figures were of intermediate value, but usually formed nodules. For best performance inoculants should probably carry 80 to 200 million viable nodule bacteria per pound of seed the culture is intended to inoculate.

LEONARD (1937) tested some inoculants supplied by agronomists of the Soil Conservation Service. The material used for observations was about six months old. Three samples of charcoal material showed no living pea or vetch organisms when tested in vegetative cultures; one sample indicated the presence of about 11 million



bacteria per g. Three samples of peat inoculants tested under similar conditions showed from 26 to 42 million rhizobia per g.

The highest number of rhizobium, obtained by HEDLIN and NEWTON (1948) was 1997 million per g of air-dry material after 14 days in a mixture of high-lime peat and black soil (with addition of nutrients), which had been sterilized beforehand. After 141 days the number of cells had decreased to 291 million per g of dry material in flasks which had been stoppered with cotton-wool. In flasks which had been closed air tight and stored at room temperature the numbers decreased to 0.

NEUBOULD (1951) obtained the highest number of *R. meliloti*, viz., 2590 million per g, in a mixture of high-lime peat and powdered charcoal to which nutrients had been added, after four months' storage at approximately 5° C. in friction sealed tinplate cans and a preliminary storage at room temperature for 24 hours to permit initial increase. The cans used, although tightly friction sealed, provided satisfactory conditions for growth as illustrated by the results given. This is not in accord with the results of HEDLIN and NEWTON, who found that all inoculants stored in closed containers, except those prepared with black soil, gave counts lower than one million per g after 38 days.

We examined a number of commercial rhizobium cultures from Sweden, made in the Baljväxtlaboratoriet Lantbrugshögskolan in Uppsala, from France (vaccinograines) sold by the Société Agrotechnique, 10 Rue Notre-Dame-de Lorette in Paris and from Germany, resp. "Azotogen" from the Institute Dr ECKOLDT in Dohna-Dresden and "Radicin" from the Radicin-Institute in Westerrade-Holstein. The data are shown in table 13.

The highest numbers of rhizobium per g of moist material were found in the Swedish cultures. The experience with these cultures in the Netherlands is generally very good.

As compared with the cultures distributed by our laboratory the numbers of rhizobia per g of moist material in these European cultures (which were determined immediately after receiving them) are much smaller (table 14).

In most cases our cultures contain, moreover, only the proper rhizobium of the cross-inoculating group in question and no other organisms. This is of importance, not only to obtain high numbers (7), but also to keep the numbers high for a long period, especially when nutrients are present, as in our case lucerne meal, for a factor

TABLE 13.

Tests of official samples of European commercial legume cultures.

| Origin         | Medium used       | Variety of legume | rhizobium in millions |          | Purity                                        |
|----------------|-------------------|-------------------|-----------------------|----------|-----------------------------------------------|
|                |                   |                   | per g                 | per acre |                                               |
| Uppsala        | Soil-peat mixture | Lucerne           | 1660                  | 94620    | Not pure:<br>530.000.000<br>foreign organisms |
| "              | "                 | "                 | 800                   | 36000    | Practically pure                              |
| "              | "                 | "                 | 2516                  | 143412   | " "                                           |
| "              | "                 | Peas              | 1096                  | 69048    | " "                                           |
| "              | "                 | Lupine            | 0                     | 0        | Only <i>Penicillium</i>                       |
| Vaccinograines | ?                 | Lucerne           | Practically none      | ?        | Not pure:<br>117.000.000<br>foreign organisms |
| Azotogen       | Sand              | Peas              | 17.2                  | 3096     | Practically pure                              |
| "              | "                 | "                 | 19.6                  | 3528     | " "                                           |
| "              | "                 | "                 | 24.0                  | 4320     | " "                                           |
| "              | "                 | Lucerne           | 10.0                  | 1800     | " "                                           |
| "              | "                 | "                 | 9.6                   | 1728     | " "                                           |
| "              | "                 | "                 | 13.6                  | 2448     | " "                                           |
| "              | "                 | Clover            | 33.2                  | 5976     | " "                                           |
| "              | "                 | "                 | 62.4                  | 11232    | " "                                           |
| "              | "                 | "                 | 45.2                  | 8136     | " "                                           |
| "              | "                 | Lupine            | 8.8                   | 1584     | " "                                           |
| "              | "                 | "                 | 18.0                  | 3240     | " "                                           |
| "              | "                 | "                 | 19.6                  | 3528     | " "                                           |
| "              | "                 | Phaseolus         | 10.8                  | 1944     | " "                                           |
| "              | "                 | "                 | 22.4                  | 4032     | " "                                           |
| "              | "                 | "                 | 20.4                  | 3672     | " "                                           |
| Radicin        | Peat-dust         | Peas              | 46.8                  | 2340     | Pure for 25-30%                               |
| "              | "                 | "                 | 56.0                  | 2790     | " " 25-30%                                    |
| "              | "                 | "                 | 67.0                  | 3300     | " " 25-30%                                    |
| "              | "                 | Lucerne           | 87.6                  | 4380     | " " 25-30%                                    |
| "              | "                 | "                 | 97.6                  | 5175     | " " 25-30%                                    |
| "              | "                 | "                 | 88.4                  | 4410     | " " 25-30%                                    |
| "              | "                 | Lupine            | 206.0                 | 10290    | " " 25-30%                                    |
| "              | "                 | "                 | 187.0                 | 9345     | " " 25-30%                                    |
| "              | "                 | "                 | 340.0                 | 16950    | " " 25-30%                                    |

that appears to influence the growth and survival of rhizobia in soil is the symbiotic or antagonistic effect of other microorganisms (1, 2, 6, 11, 17, 19, 20, 24).

TABLE 14.

Comparison of the numbers of rhizobia in some European cultures with cultures distributed by the laboratory in Kampen (numbers in millions per g of moist material).

| Variety of legume    | Cultures from Uppsala | Azotogen  | Radicin   | Cultures from Kampen |
|----------------------|-----------------------|-----------|-----------|----------------------|
| Lucerne              | 800—2516              | 9.6—13.6  | 87.6—97.6 | 10000—20000, or more |
| Peas                 | 1096                  | 17.2—24.0 | 46.6—67.0 | 4500—9000            |
| Clover               | —                     | 33.2—62.4 | —         | 4000—15000           |
| Phaseolus            | —                     | 10.8—22.4 | —         | 5000—10000           |
| Lupine               | —                     | 8.8—19.6  | 187—340   | 13000—18000          |
| g of medium per acre | 40—48                 | ± 180     | ± 45      | ± 60                 |

## MODE OF APPLICATION.

As already mentioned the cultures are prepared in dispensing-bottles, which are half filled with the medium. When the culture is ready, it is not removed from the bottle until it is used for the inoculation of the legume seed. The use of the dispensing-bottles has the advantage that impurities by molds can be detected immediately. Moreover they are cheap, don't rust and don't break easily. Before use they are filled for 4/5th part with water, closed with one hand and shaken vigorously for two minutes. The suspension is mixed afterwards with the seeds.

Most of the bacteria are present in the water after shaking for two minutes, as could be demonstrated as follows:

A number of suspensions were made from 100 g of a culture with 400 ml water in a glass vessel of 600 ml:

- The suspension was stirred for 2 minutes with a stirring-rod. After settling for 1 minute, 10 ml of the solution was pipetted at a distance of 5 cm above the deposit.
- After addition of some water, the mash was kneaded for 2 minutes by hand. Then more water was added to make up 400 ml. The suspension was quickly stirred and after settling for 1 minute 10 ml of the solution was pipetted as in *a*.
- As *b*, but the mash was kneaded for 5 minutes.
- As *b*, but the mash was kneaded for 10 minutes.

- e. The suspension was quickly stirred, this was repeated after 10 minutes and after settling for 1 minute, 10 ml were pipetted as in *a*.
- f. After adding 400 ml water the mixture was treated for 45 minutes in a ball-mill and immediately afterwards 10 ml were taken from the suspension with a pipette.

The pipetted suspensions contained the following numbers of rhizobia per ml:

|                       |               |
|-----------------------|---------------|
| Suspension <i>a</i> : | 2.774.000.000 |
| „ <i>b</i> :          | 2.526.000.000 |
| „ <i>c</i> :          | 2.914.000.000 |
| „ <i>d</i> :          | 2.574.000.000 |
| „ <i>e</i> :          | 2.056.000.000 |
| „ <i>f</i> :          | 2.154.000.000 |

From these numbers we can conclude that no more bacteria are released by kneading, or by treating in a ball-mill, than by simple stirring for 2 minutes.

#### FIELD EXPERIMENTS.

The efficiency of our cultures was tested in 1950 and 1951 in the North-Eastern polder. On section E 77 two experimental fields were laid out, on which uninoculated *Medicago lupulina* was compared in triplicate with *M. lupulina* of which one kg of seed had been inoculated resp. with 0.5, 1, 2, 4, 8 and 16 g of inoculant. On the first experimental field sowing occurred on 4th May in a dry period. On this field by the middle of July a slight difference in colour was detectable between the plots with 0.5, 1, 2 and 4 g of inoculant. The plots with 4, 8 and 16 g did not show any differences. At the beginning of August a clear difference in growth and colour was detectable only between the uninoculated and inoculated plots. No differences were to be seen between the inoculated plots at this time. The inoculant used in this trial contained 10650 millions rhizobia per g.

On the second experimental field the sowing took place at 15th June in a rainy period. On this field no differences in the development or colour were detected between the inoculated plots: 0.5 g of inoculant per kg of seed had the same effect as 16 g of inoculant. There was a marked difference, however, in growth and colour between the inoculated and uninoculated plots. This means that in this case 10 g of the inoculant for 20 kg of seed, or 4 g per acre,



have given a quite satisfactory result. This inoculant, which had been stored for some time at room temperature, contained 8700 million rhizobia per gram.

In 1950 the effect of an inoculant, made in our laboratory for lucerne, was compared in triplicate on section C 131 with the German cultures Radicin and Azotogen. The 3 cultures were used in quantities corresponding with resp. 45, 45 and 180 g per acre. The cultures contained resp. about 10.000 million, 90 million and 11 million rhizobia per g of moist material. The plots were sown on July 21. On 22nd August a number of plants from each plot were examined for the presence of nodules. The following relative numbers of nodules were found:

|                                        |      |
|----------------------------------------|------|
| 1: Treated with the culture of Kampen: | 100% |
| 2:   "      "      Radicin             | 85%  |
| 3:   "      "      Azotogen            | 15%  |
| 4: Uninoculated                        | 10%  |

On that day all plants on plot 1 showed a perfectly healthy growth. This was also the case with most of the plants on plot 2, with exception of a small percentage that had a yellowish tinge and a less good development. There was only a slight difference in favour to the plants on plot 3 in comparison with those on plot 4, which stood markedly behind in growth and showed a yellow green appearance. Gradually the difference between plots 1 and 2 disappeared. After two months they were practically absent.

The wind-dry yields of the first cut were as follows: plot 1: 39.1 kg; plot 2: 38.0 kg; plot 3: 18.8 kg and plot 4: 14.6 kg, or calculated as percentages of the yield on plot 4, resp. 268, 260, 128 and 100 %.

Thus in this case our culture and the Radicin culture have given practically the same results, though they differed markedly in numbers of rhizobia. The Azotogen culture proved to be of inferior quality. In this connection it may be mentioned that LEMMERMAN (1947) and SCHMIDT (1948) noted unsatisfactory results with German inoculants. LEMMERMAN regrets that such inoculants are sold without giving the farmer any security of a good effect.

In 1951 some experimental fields were set out on section E 77 in co-operation with the Agricultural Section (Head: Ir W. A. BOSMA). On the first field lucerne was sown on the 2nd May, *viz.*, the species Beaux à haute tige, Chartain Villiers, du Puits, Provencer and Grimm. The effect was compared in triplicate of soil-peat cultures of Kampen, soil-peat cultures of Uppsala and of liquid cultures,

prepared with the strains of *R. meliloti* A 15, A 42, A 48, A 55 and A 84. The soil-peat cultures were used in quantities used in practice, the liquid cultures in overdose. The strains A 15 and A 42 are used in our laboratory for 20 years for the Zuiderzee polders. Strain A 48 had been isolated from Radicin, A 55 from Azotogen. Strain A 84, originally *R. meliloti* strain A.H. of the Rothamsted Experimental Station, had been kindly supplied by Dr H.G. THORNTON on 3-2-1951. The same strains were tested in tubes in the greenhouse.

Strain A 55 did not react at all, as well in the field as in the greenhouse. Therefore only the results with the other cultures are shown in table 15.

TABLE 15.

Yields of the first cut (hay with 15% moisture) in kg per are from the experimental field on section E 77 (fields 36, 37 and 38), sown on 2nd May 1951.

| Lucerne species             | Not in-oculated | Cultures of Kampen | Cultures of Uppsala | Strain A 48 | Strain A 15 | Strain A 42 | Strain A 84 |
|-----------------------------|-----------------|--------------------|---------------------|-------------|-------------|-------------|-------------|
| Beaux à haute tige Chartain | 31.6 ± 4.0      | 45.6 ± 4.5         | 42.0 ± 5.3          | 40.8 ± 2.1  | 43.0 ± 4.9  | 45.4 ± 5.0  | 44.9 ± 2.3  |
| Villiers                    | 31.0 ± 3.9      | 40.8 ± 3.8         | 37.5 ± 1.9          | 38.9 ± 1.5  | 38.3 ± 1.2  | 39.9 ± 0.9  | 39.9 ± 0.7  |
| Flandria                    | 31.5 ± 1.0      | 43.3 ± 1.3         | 43.3 ± 1.3          | 41.7 ± 4.0  | 41.3 ± 1.3  | 42.0 ± 4.2  | 46.7 ± 0.9  |
| du Puits                    | 36.5 ± 0.7      | 48.0 ± 4.7         | 46.4 ± 4.7          | 44.3 ± 4.1  | 45.1 ± 3.3  | 46.3 ± 5.6  | 47.6 ± 4.8  |
| Grimm                       | 29.7 ± 2.2      | 43.1 ± 3.1         | 41.4 ± 1.7          | 41.5 ± 5.4  | 44.3 ± 2.6  | 44.3 ± 0.5  | 45.5 ± 5.3  |
| Provencer                   | 33.7 ± 0.7      | 43.3 ± 0.9         | 42.7 ± 4.1          | 41.2 ± 2.0  | 44.9 ± 4.5  | 44.4 ± 3.5  | 43.1 ± 2.2  |

Data on protein contents and protein production, which were determined in some cases, are presented in table 16.

TABLE 16.

Data on protein contents and protein production of the first cut of some lucerne species from the experimental field E 77, sown on 2nd May 1951.

| Lucerne species | Not in-oculated |        | Cultures of Kampen |        | Cultures of Uppsala |        | Strain A 15 |        | Strain A 42 |        | Strain A 84 |        |
|-----------------|-----------------|--------|--------------------|--------|---------------------|--------|-------------|--------|-------------|--------|-------------|--------|
|                 | %               | kg/are | %                  | kg/are | %                   | kg/are | %           | kg/are | %           | kg/are | %           | kg/are |
| du Puits        | 16.1            | 4.9    | 17.5               | 7.1    | 15.9                | 6.3    | 18.6        | 7.1    | 16.4        | 6.5    | 16.9        | 6.8    |
| Grimm           | 18.3            | 4.6    | 19.6               | 7.2    | 19.1                | 6.7    | 18.9        | 7.1    | 18.6        | 7.0    | 18.8        | 7.3    |
| Provencer       | 15.7            | 4.5    | 17.9               | 6.6    | 17.3                | 6.3    | 16.4        | 6.3    | 17.3        | 6.5    | 18.9        | 6.9    |

The data in table 15 show that in most cases the cultures of Kampen have given a somewhat higher yield than the cultures of Uppsala. The highest yields were obtained with the strains A 15, A 42 and A 84. For Flandria strain A 84 proved to be substantially the best one. It may be stated here that this strain gave also very good results on an experimental field carried out in 1951 with black medic, as compared with our strains of *R. meliloti* for this crop.

As regards protein contents and protein production in the three tested legume species presented in table 16, the cultures of Kampen have given somewhat better results than those of Uppsala. Strain A 84 produced a little more protein than strain A 15, except in the variety du Puits and also a little more than strain A 42.

On two other experimental fields on section E 77, on the fields no. 40 and 41 soil-peat cultures of Kampen were compared in triplicate with those of Uppsala in a number of lucerne species. Sowing on these fields was carried out on 5th June and on 3rd July 1951, respectively. The yields of the first cut are given in table 17.

TABLE 17.

Yields of the first cut (hay with 15% moisture) in kg per are from the fields no. 40 and 41 on section E 77.

| Treat-<br>ment                           | Beaux<br>à haute<br>tige | Chartain<br>Villiers | du<br>Puits | Flandria   | Grimm      | Proven-<br>cer | Frän-<br>kische <sup>1)</sup> |
|------------------------------------------|--------------------------|----------------------|-------------|------------|------------|----------------|-------------------------------|
| Sown on<br>5-6-1951<br>uninocu-<br>lated | 23.7 ± 2.2               | 22.8 ± 0.9           | 24.9 ± 0.5  | 24.7 ± 0.4 | 21.4 ± 5.2 | 21.1 ± 1.4     |                               |
| Cultures of<br>Kampen                    | 33.3 ± 1.7               | 27.1 ± 1.4           | 33.2 ± 2.2  | 31.8 ± 0.9 | 29.8 ± 0.8 | 29.0 ± 0.3     |                               |
| Cultures of<br>Uppsala                   | 32.6 ± 2.5               | 29.0 ± 2.3           | 32.3 ± 1.1  | 30.1 ± 1.0 | 28.9 ± 0.3 | 30.0 ± 1.0     |                               |
| Sown on<br>3-7-1951<br>uninocu-<br>lated | 24.7 ± 1.6               | 20.7 ± 1.7           | 25.3 ± 3.3  | 24.2 ± 2.8 | 19.0 ± 1.4 | 21.9 ± 1.1     | 21.9 ± 0.2                    |
| Cultures of<br>Kampen                    | 28.3 ± 1.7               | 26.6 ± 5.0           | 31.3 ± 3.6  | 27.4 ± 2.6 | 22.6 ± 2.0 | 26.0 ± 1.7     | 27.5 ± 0.0                    |
| Cultures of<br>Uppsala                   | 25.5 ± 0.8               | 24.7 ± 4.0           | 30.9 ± 5.3  | 26.8 ± 3.7 | 23.1 ± 1.7 | 26.0 ± 1.7     | 24.9 ± 0.5                    |

<sup>1)</sup> in duplicate.

In some cases protein contents and protein production were determined. These data are shown in table 18.

TABLE 18.

Data on protein contents and protein production of the first cut of some lucerne species from an experimental field on section E 77, field no. 40, sown on 5th June 1951.

| Légume species | Uninoculated |        | Cultures of Kampen |        | Cultures of Uppsala |        |
|----------------|--------------|--------|--------------------|--------|---------------------|--------|
|                | %            | kg/are | %                  | kg/are | %                   | kg/are |
| du Puits       | 18.1         | 4.51   | 21.6               | 7.17   | 21.7                | 7.01   |
| Grimm          | 19.0         | 4.06   | 23.4               | 6.96   | 23.5                | 6.79   |
| Provencer      | 16.0         | 4.38   | 21.4               | 6.21   | 21.1                | 6.33   |

From the data in the tables 15—18 it may be concluded that, though the differences were very small and not always significant, there is a tendency for the cultures from Kampen to give a slightly higher yield of hay and protein than those from Uppsala on slightly alkaline soils of the North-Eastern Polder.

The field experiment with Radicin showed, that satisfactory results have been obtained with a culture, containing 90 million rhizobia per g, corresponding with 4050 million per acre. In comparison with this inoculum the numbers in our cultures, *viz.*, 4000—20.000 million per g, or 240.000—1200.000 million per acre, are extremely high. These numbers are also high in comparison with other foreign cultures. This means, that a large number of the rhizobia in our inoculants may succumb, before the inoculants lose their value. This fact and also the fact that the numbers remain high for a long period, when the cultures are kept at 2° C., ensure a good product being maintained for a long time.

### Summary.

A study was made of the growth of rhizobia in mixtures of peat, heavy loam and  $\text{CaCO}_3$ , with or without addition of salts and of organic nutrients. Extremely high numbers (4000—20.000 millions per g of moist material) were obtained by using alder bog peat and 10—20 % lucerne meal in these mixtures and by growing the rhizobia in pure condition with access of air. When lucerne meal is



present in these quantities, addition of salts, yeast and other nutrients is superfluous. Good results have been obtained by using dispensing-bottles as containers, plugged with cotton-wool. The media in the bottles are inoculated with a liquid inoculum by means of a hollow needle, which is stuck between the cotton-wool plug and the wall of the neck of the bottle. By this simple procedure contamination of the media with air-borne organisms is reduced to a minimum. The numbers remain high for a long period when the cultures are stored at 2° C. with access of some air.

### Acknowledgement.

We are much indebted to Dr H. G. THORNTON, Head of the Soil Microbiology Department (Rothamsted Experimental Station) for being so kind to read the manuscript and whose criticism was very helpful.

### Literature.

1. CASAS CAMPILLO, C. 1947. *Cieniaa* **8**, 108.
2. DUGGAR, B. M. and PRUCHA, M. J. 1912. *Science* **35**, 229.
3. ERDMAN, L. W. 1948. *Farmer's Bull.* No. **2003**, 20 pp.
4. FELLERS, C. R. 1918. *Soil Sci.* **6**, 53.
5. GERRETSEN, F. C. 1950. *Maandbl. v. d. Landb. Voorlichtingsdienst.* **7**, 16.
6. ABDEL-GHAFFAR, A. S. and ALLEN, O. N. 1950. *Fourth Intern. Congr. of Soil Sc., Transact.* **3**, 93.
7. HEDLIN, R. A. and NEWTON, J. D. 1948. *Canad. Jour. of Res.* **26**, 2.
8. HOVER, A. W. and CONN, H. J. 1931. *New York State Agr. Exp. Sta. Bull.* **602**.
9. HOVER, A. W. 1938. *Jour. Am. Soc. of Agr.* **30**, 451.
10. KNIGHT, H. G. 1930. *U.S. Dept. Agr. Bur. Chem. and Soils.* 50 pp.
11. LANDERKIN, G. B. and LOCHHEAD, A. G. 1948. *Canad. Jour. of Res.* **26**, 501.
12. LEMMERMAN, O. 1947. *Zeitschr. f. Pfl. Düng. u. Bodenk.* **38**, 80.
13. LEONARD, L. T. 1937. *Soil Sci. Soc. Am. Proc.* **2**, 305.
14. LEONARD, L. T. 1944. *U.S. Dept. of Agr. Circ. No.* **703**, 8 pp.
15. NEWBOLD, F. H. S. 1951. *Scientific Agr.* **31**, 463.
16. NOBBE, F. und HILTNER, L. 1896. *Sächs. Landw. Zeitschr.* **44**, 90.
17. NOBBE, F. und HILTNER, L. 1899. *Landw. Vers. Stat.* **51**, 447.
18. ROBISON, R. S. 1945. *Soil Sci. Soc. Amer. Proc.* **10**, 206.
19. ROBISON, R. S. 1946. *J. Bact.* **51**, 129.
20. SACKETT, W. G. 1906. *Mich. Acad. Sci.* **8**, 147.
21. SCHMIDT, O. C. 1948. *Zeitschr. f. Pfl. Düng. u. Bodenk.* **42**, 268.
22. VAN SCHREVEN, D. A., HARMSSEN, G. W., LINDENBERGH, D. J. and OTZEN, D. 1953. *Antonie van Leeuwenhoek* **19**, 300.
23. TEMPLE, J. C. 1916. *Ga. Agr. Expt. Sta. Bull.* **120**, 65.
24. THORNTON, G. D., ALENCAR, J. and SMITH, F. B. 1949. *Soil Sci. Soc. Amer. Proc.* **14**, 188.

(Cornell University, Geneva, New York).

## THE CLASSIFICATION OF LUMINESCENT BACTERIA

by

**ROBERT S. BREED** and **ERWIN F. LESSEL, Jr.**

(Received July 21, 1953).

In preparing the various editions of *BERGEY's Manual of Determinative Bacteriology*, it has become increasingly evident that the classification of the luminescent bacteria has not been properly developed either in the Manual or anywhere else. A careful review of the literature has been made with the active assistance of Professor A. J. KLUYVER of Delft, Holland whose firsthand knowledge of these bacteria has been invaluable. Professor HARVEY's books (1940, 1952) which deal with bio-luminescence in all living things include excellent bibliographies and have also been very helpful. The purpose of this paper is to develop the classification of luminescent bacteria, as it is found in the literature, in accordance with accepted principles of nomenclature and taxonomy.

A review of the published descriptions of species of luminescent bacteria gives good reason for thinking that all of these organisms are Gram-negative, non-sporeforming and polar flagellate when motile. These characteristics definitely place the luminescent bacteria as members of either the Family *Pseudomonadaceae* or the Family *Spirillaceae*. However, in the past these species have been placed in as many as 15 different genera, eight of which belong in families other than the two mentioned above. These genera are as follows: *Micrococcus*, *Coccobacillus*, *Sarcina*, *Bacillus*, *Bacterium*, *Photobacterium*, *Photobacter*, *Arthrobacterium*, *Vibrio*, *Microspira*, *Photospirillum*, *Photomonas*, *Pseudomonas*, *Pasteurella* and *Achromobacter*.

Luminescent bacteria are commonly found on or in dead or living animals as they occur on sea beaches, in sea water or living symbiotically in luminous organs of cephalopods and deep sea fishes. They may even be pathogenic to the animals in which they are found. Occasionally such bacteria have been described as occurring in fresh water shrimp (Japan), insect larvae, meat as found in butcher shops, even in human flesh wounds or other sources that are not associated with salt water. While they do not normally occur on plant materials in nature, they do grow on potato media and have been found growing naturally on potatoes.

All of the bacteria discussed in this paper possess a unique ability to produce light and are commonly described as phosphorescent, a term that implies that they absorb light energy, later releasing it when in the dark. They are, however, more properly described as luminescent, a term that indicates that they produce their own light. This ability may be lost just as virulence is sometimes lost on continued artificial cultivation of pathogenic bacteria; but this ability is normally regained rather readily if the organisms are cultivated on suitable media. This frequently means cultivation on neutral or slightly alkaline media prepared from sea water or containing equivalent amounts of the required salts. The majority of species come from marine habitats.

The stability of this luminescent character led to a suggestion by BEIJERINCK (1889) that all luminescent bacteria should be placed in a single Genus, *Photobacterium*. Four species were placed in the genus: *P. phosphorescens* Beijerinck, syn. *Bacterium phosphorescens* Fischer (1888); *P. indicum* Beijerinck, syn. *Bacillus phosphorescens* Fischer (1887); *P. fischeri* Beijerinck, described by FISCHER (1888) as the 'Einheimischer Leuchtbacillus', and *P. luminosum* Beijerinck, a new species found by the latter author in sea water. Because the characterization of *Photobacterium* by BEIJERINCK was chiefly based on physiological characters and because he disregarded important differences in the morphology of the four species, some have thought this name unusable. However, ENLWS (1920) designated *Photobacterium phosphorescens* Beijerinck as the type species of the genus. With a proper recognition of this fact and a suitable emendation of the description given by BEIJERINCK, this name that has priority becomes entirely legitimate and appropriate. Such a characterization of the genus follows:

*Photobacterium* Beijerinck, 1889 char. emend.

(BEIJERINCK, Arch. Néerl. d. Sci. Exact. et Natur., **23**, 401, 1889; *Photobacter* Beijerinck, Proc. Sect. Sci., Kon. Akad. v. Wetensch. Amsterdam **3**, 352, 1900).

Coccobacilli and rods which, in the presence of glucose and asparagine, may develop into bacteroids. Polar flagellate when motile. The type species is normally non-motile but shows motility in young culture (KLUYVER). May or may not liquefy gelatin. Produce acid, or acid and visible gas ( $H_2$  and  $CO_2$ ), from glucose and other sugars. Usually produce no acid or gas from lactose. Aerobic, facultative anaerobic. Luminescent. Growth and luminescence best, or even exclusively, on salt water media. Found on dead fish and other salt water animals, and in sea water. Reports by various authors indicate that the luminescent, coccoid and rod-shaped bacteria found living symbiotically in the tissues of the luminous organs of various cephalopods and deep sea fishes belong to this genus. Other coccoid and rod-shaped luminescent bacteria found in the blood of crustacea and caterpillars appear to be parasitic or even pathogenic.

The Genus *Photobacterium*, as defined above, includes not only the type species, *Photobacterium phosphorescens* Beijerinck, but also two other generally recognized species. Quite probably additional species should be recognized. Among numerous species of luminescent bacteria that have been described, more than forty will be listed in the seventh edition of BERGEY's Manual as having characters that indicate that they are either identical with or closely related to the three species placed in this genus. Two of the four species originally placed in *Photobacterium* by Beijerinck are excluded from the genus by the definition as given here.

Practically every student that has made a comprehensive study of luminescent bacteria has reported finding bacteria having characters like those of the type species of *Photobacterium* no matter whether the material was collected off the shores of European countries (Germany, Holland, France, Russia, Italy) where the earliest studies were made, or in Australia, Java, Japan, eastern United States of America or the West Indies. As cultures of the type species are usually non-motile and as the type species is so nearly spherical in form that it was called a micrococcus in early days (later coccobacillus), it has been difficult to determine its relationship to other bacteria. Other characters indicate, however,

that this species should be placed in the Genus *Photobacterium* Beijerinck in the same family as the Genus *Pseudomonas*.

Two other well described, polar flagellate, more definitely rod-shaped species are also included in this genus. The first of these, *Photobacterium fischeri*, is a species that was included in the genus by BEIJERINCK in 1889. It is more completely characterized in recent papers by JOHNSON *et al.* (1936, 1943). Several photomicrographs of cells with definite tufts of flagella are reproduced in the latter paper. The cells vary in form from coccoid to definite rods, practically all of which are straight, not curved.

JOHNSON and SHUNK (1936) also describe a similar monotrichous, luminescent bacterium under the name *Achromobacter harveyi*. As *Achromobacter* is now defined as a genus where the organisms are peritrichous when motile, this species was transferred to the Genus *Pseudomonas* in the sixth edition of BERGEY'S Manual (1948). It is now felt that it should be placed in the Genus *Photobacterium* Beijerinck under the name *Photobacterium harveyi* comb. nov.

The fermentation of glucose by these species appears to be carried out in the same way that species now placed in the Family *Enterobacteriaceae* carry out this fermentation. As in the case of paracolon bacteria, proper tests of some of these luminescent bacteria may even show a delayed fermentation of lactose with the possible production of acid and gas. The chief differences between the species placed in *Photobacterium* and the species placed in the Family *Enterobacteriaceae* lie in the polar attachment of the flagella and in the luminescent property of the species placed in *Photobacterium*. *Photobacterium* Beijerinck should be placed near the Genus *Aeromonas* Kluyver and van Niel as the latter genus also includes polar flagellate species that appear to ferment glucose much as do the peritrichous species in the Family *Enterobacteriaceae*. While it may be a matter of no significance from the standpoint of establishing relationships, it is interesting that the species placed in the Genus *Aeromonas* commonly cause diseases of fishes while those placed in the related Genus *Photobacterium* are very commonly found on dead fish.

It is clear that two of the four species placed by BEIJERINCK in his Genus *Photobacterium* would now be regarded as vibrios. This became evident as early as 1896 when LEHMANN and NEUMANN placed *Photobacterium indicum* and *P. luminosum* in the Genus *Vibrio*. Then MIGULA (1900) placed *P. luminosum* in the Genus



*Microspira*, the genus proposed by SCHROETER (1886) to include the cholera and other vibrios. A year later CHESTER (1901) placed both of the species named above in *Microspira*.

Another luminescent vibrio that attracted early attention was DUNBAR's vibrio, isolated from Elbe River water during an attempt to isolate the cholera vibrio (1893). The description of this species merely states that it is indistinguishable from the cholera vibrio except for the fact that it is luminescent under proper cultural conditions and that it appears to be harmless to man. Later it was named *Vibrio albensis* Lehmann and Neumann (1896). Another interesting species isolated from the photogenic organ of a squid appears to deserve recognition. This is *Bacillus pierantonii* Zirpolo (1918), syn. *Vibrio pierantonii* Meissner (1926). While the above-mentioned species of *Vibrio* will be described in full in the forthcoming edition of BERGEY's Manual, about 12 additional species that appear to be vibrios will also be listed. Some of these appear to be identical with species that were described earlier. Comparative studies of cultures must be made before any satisfactory arrangement of these species can be prepared.

Consideration was given to a revival of the invalidly published generic name proposed by MIQUEL and CAMBIER (1902) in a binomial in the following form, *Vibrio dunbari* (*Photospirillum dunbari*). However, as these authors did not accept and use *Photospirillum* and as the organisms are really very closely related to the type species of the genus, *Vibrio*, they are retained in the Genus *Vibrio*.

A comment may be made here on the use of the specific epithet "phosphorescens" that may be helpful to others who attempt to review the story of luminescent bacteria. The widespread use of this epithet in the literature for several different organisms has led to much confusion.

BEIJERINCK (1889) was forced to propose a new specific epithet for one of FISCHER's species when he placed them both in the same genus. Thus he retained the specific epithet 'phosphorescens' for the species named *Bacterium phosphorescens* by FISCHER and proposed the specific epithet "indicum" for the species named *Bacillus phosphorescens* by FISCHER. The specific epithet "phosphorescens" was validly published and is legitimate in the combination *Photobacterium phosphorescens* (Fischer) Beijerinck and should continue in use.

BEIJERINCK (1916) discussed the confusion that had arisen by

that time. In a footnote he himself made the error of attributing this confusion to LEHMANN and NEUMANN. He mentions the description of *Bacterium phosphorescens* Fischer as given by LEHMANN and NEUMANN on p. 316 of the fifth edition of their *Bacteriologische Diagnostik* (1912) as if it were their description of *Photobacterium indicum*. However, LEHMANN and NEUMANN's description of the latter species (*Bacillus phosphorescens* Fischer) is found on p. 530 where it is properly described as *Vibrio indicus*.

The real trouble appears to have arisen from the fact that FISCHER (1887) first described *Bacterium phosphorescens*, without naming it, in an appendix to his 1887 paper. Then BAUMGARTNER (1887) in the same year named this unnamed species *Bacillus phosphorescens* II while FISCHER named it *Bacterium phosphorescens* the following year. Later authors frequently refer to FISCHER's 1887 paper as giving the original description of this species without indicating that the organism was not named until the following year. The organism that was named in the 1887 paper is *Bacillus phosphorescens*.

In more recent times, *Bacillus phosphorescens*, *Bacterium phosphorescens*, *Vibrio phosphorescens* and other binomials with the specific epithet "phosphorescens" have been applied almost indiscriminately to any culture of a luminescent bacterium regardless of any relationship it might have to the organisms first given this specific epithet. The specific epithet "indicum" has meanwhile retained its original meaning without any confusion that we have discovered.

### S u m m a r y.

The luminescent bacteria are logically placed in two genera. The common coccoid and frequently non-motile species placed by BEIJERINCK first in his genus *Photobacterium*, 1889 under the name *Photobacterium phosphorescens* syn. *Bacterium phosphorescens* Fischer, should be recognized as the type species of *Photobacterium*. Other characters indicate that this genus should be placed in the Family *Pseudomonadaceae* Winslow *et al.* and should include other straight, rod-shaped, luminescent, polar flagellate bacteria that ferment glucose without, however, necessarily producing gas ( $H_2$  and  $CO_2$ ) as does the type species. The species that have the form of vibrios should be accepted as members of the genus *Vibrio* as suggested

by several previous investigators. They have characters much like those of *Vibrio comma*, the type species of the genus *Vibrio*.

### References.

- VON BAUMGARTEN, P. 1887. Baumgarten's Jahresbericht **3**, 344.  
BEIJERINCK, M. W. 1889. Arch. Néerl. d. Sci. Exact. et Nat. **28**, 401.  
BEIJERINCK, M. W. 1916. Folia Microbiologica **4**, 15, footnote 4.  
BREED, R. S., MURRAY, G. D. and HITCHENS, A. P. 1948. Bergey's Manual of Determinative Bacteriology. 6th. Ed., p. 110.  
CHESTER, F. D. 1901. Manual Determinative Bacteriology. New York, p. 333—334.  
DUNBAR, W. 1893. Deutsch. med. Wochenschr. **19**, 799.  
ENLOWS, E. M. A. 1920. Hyg. Lab. Treasury Dept., U.S.P.H. Service, Bull. **121**, 71.  
FISCHER, B. 1887. Z. f. Hyg. **2**, 158.  
FISCHER, B. 1888. Centralbl. f. Bakt. **3**, 107.  
HARVEY, E. N. 1940. Living Light. Princeton Univ. Press.  
HARVEY, E. N. 1952. Bioluminescence. Academic Press, New York.  
JOHNSON, F. H. and SHUNK, I. V. 1936. J. Bact. **31**, 585.  
JOHNSON, F. H., ZWORYKIN, N. and WARREN, G. 1943. J. Bact. **46**, 167.  
LEHMANN, K. B. und NEUMANN, R. O. 1896. Bakteriologische Diagnostik. München. 1. Aufl., 2, 340—341.  
MEISSNER, G. 1926. Centralbl. f. Bakt. II. **67**, 200.  
MIGULA, W. 1900. System der Bakterien. Jena, **2**, 1015 and 1013.  
MIQUEL, P. et CAMBIER, R. 1902. Traité de Bactériologie. Paris. p. 886.  
SCHROETER, J. 1886. in: Cohn, Kryptogamen-Flora von Schlesien **3**, 168.  
ZIRPOLO, G. 1918. Boll. nat. Napoli. **30**, 206.
-

(From the Department of Bacteriology and Experimental Pathology of the Netherlands' Institute for Preventive Medicine, Leiden).

## BIOCHEMICAL PROPERTIES AND THE PRODUCTION OF TOXINS AND ENZYMES BY STAPHYLOCOCCI ISOLATED FROM CASES OF SECONDARY PNEUMONIA FOLLOWING INFLUENZA

by

**H. A. E. VAN TONGEREN**

(Received April 18, 1953).

Since the varieties of *Micrococcus pyogenes* (staphylococci) belong to the group of ubiquitous microorganisms they can occasionally give rise to difficulties in the assignation of the pathogenic agent, for strains exist which appear to be either pathogenic or apathogenic for man. The sense of the word pathogenic as applied to staphylococci includes every strain which has the capacity of giving rise to an infection or a food intoxication in man under optimal conditions. An attempt is generally made to distinguish the pathogenic and non-pathogenic groups by using one or more of the *in vitro* methods which can be carried out quite easily as routine work. General opinion, however, concerning the value of these methods as indicators is still rather controversial. The formation of a certain toxin or enzyme by a staphylococcal strain is considered by one investigator as being an "accurate and convenient criterion", another, however, can be found to say it is an "imperfect index" of pathogenicity..

On using an *in vivo* reaction it is generally considered that a staphylococcal strain is pathogenic when, after inoculating the culture-filtrate into the skin of a rabbit or guinea pig a dermo-necrotic reaction results, or if it kills a rabbit after parenteral inoculation within a certain period of time. Of the *in vitro* reactions the coagulase test is widely recognized as being a useful indication of pathogenicity, while other workers attach more significance to the formation of haemolysins. Some investigators have found complete correlation between the latter reactions; others have not. As we cannot yet rely upon a single biochemical reaction to determine pathogenicity it is preferable to carry out as many tests as possible, for it has become evident that the chance of a strain possessing pathogenic properties is in proportion to the number of positive *in vivo* tests.

Several workers have carried out comparative tests with large numbers of staphylococcal strains by using a few *in vitro* reactions, sometimes combined with one *in vivo* reaction. The present paper is a report concerning the activities of a small number of staphylococcal strains. All the available tests have been performed and are mutually compared. No attempt is made, however, to solve the conflict pathogenic — non-pathogenic, but the results would indicate the advisability of undertaking a more extensive study, using the more reliable and applicable *in vitro* methods.

In an experimental investigation on the influence of secondary staphylococcal infections, in the mouse-lung infected with influenza virus, twelve staphylococcal strains have been used (VAN TONGEREN, 1951). These strains were isolated from the sputum or from the lungs of patients suffering or having died from a secondary staphylococcal pneumonia following influenza. It seemed desirable to inquire into the biological activities of the microorganisms used in these experiments, as far as possible at the time (1949), in order to check whether a correlation existed between these activities and the observed pathogenicity for mice.

#### BIOCHEMICAL REACTIONS.

Before investigating the extent to which the staphylococcal strains produced exotoxins and other substrates (enzymic and non-toxic substances) their biochemical behaviour was established in a large number of media. All strains formed acid (but no gas) from glucose, lactose, maltose, mannitol, sucrose, galactose, fructose and glycerol; salicin, sorbitol, inositol, raffinose,



arabinose, aesculin and inulin were not fermented. All strains produced acid, clotted and peptonized litmus milk; the methyl-red test, the Voges-Proskauer reaction, the reduction of nitrates to nitrites, the production of ammonia and the liquefaction of gelatin were positive in all strains. None of the strains produced indole or hydrolysed starch, but all strains demonstrated a strong lipolytic activity, as determined by the hydrolysis of fats on the fat-plate of EYKMAN (1901) and on the modified "cream-plate" of ORCUTT and HOWE (CHRISTIE and GRAYDON, 1941). Of these twelve strains eleven belonged to the species *Micrococcus pyogenes* var. *aureus* and one to the species *M. pyogenes* var. *albus* (strain 7).

All the above mentioned staphylococcal strains thus exhibit great metabolic activity, which is more frequently observed in pathogenic than in non-pathogenic varieties, although too much significance may not be attached to these reactions as a means of identification.

### STAPHYLOTOXINS.

The toxins are dealt with in the following sequence of the time in which they have been described first, without due emphasis on their importance as a means of differentiation. Although in most articles one medium is given on which one can obtain the maximum production of any toxin, it appeared during these experiments that this does not hold for all toxins equally; because of this each toxin is discussed separately and, if necessary, mention will be made of the medium in which an optimal production was secured.

### LEUCOCIDIN.

The first description of a bacterial leucocidin was given in 1894 (VAN DE VELDE). To demonstrate this water soluble toxin *in vitro*, NEISSER and WECHSBERG (1901) developed a bioscopic method, based on the property that fresh intact rabbit leucocytes reduce a weak solution of methylen blue under partial anaerobic conditions, whereas lysed leucocytes do not. Since this method presented a number of difficulties PANTON and VALENTINE developed a microscopic method, between 1932 and 1939, in which lysed polymorphonuclear leucocytes of man or rabbit could be observed microscopically in a stained smear. The preparation and estimation of smears often present great difficulties and much experience is required; for these reasons the method is never used in routine work.

In 1942 another bioscopic test was developed by TODD which was based upon the observation that intact leucocytes suspended in saline will clot on the addition of a solution of caustic soda. The leucocidins of streptococci have been particularly investigated by TODD and they only appear to act on leucocytes under reduced conditions. The demonstration of leucocidins of staphylococci has the advantage that these special measures are not necessary.

Some difficulties arose in obtaining a sufficiently high leucocidin production. When the pH in the medium rose above 8 or fell lower than 6, the toxin either became rapidly inactive or deteriorated. Furthermore it appeared that on adding phosphates (buffer), glucose or serum to the medium these substances interfered with the reading of the reaction. Peptone "Witte" gave a poor toxin production. The best results were obtained with a medium of the following composition:

|                     |     |                                    |
|---------------------|-----|------------------------------------|
| Veal infusion-broth | 100 | ml (without the addition of salts) |
| Proteose peptone    | 2   | g                                  |
| Agar                | 0.6 | g      pH = 7.0                    |

Plates of this medium were inoculated with staphylococci and placed in the incubator for 4 to 6 days at 37°C. in an atmosphere containing 15 to 20% CO<sub>2</sub>. After this period the medium was centrifuged (5.000 rpm) the supernatant harvested and again centrifuged (1/4 hr. 15.000 rpm). The supernatant obtained from the latter was used as toxin. This toxin was diluted with saline (pH = 7.2—7.4) for titration purposes. Todd diluted the toxin with a mixture of equal amounts of saline and peptone water, but in our experiments the addition of the peptone water gave difficulties in reading the reactions.

Since rabbit leucocytes could not be used (because of the presence of  $\alpha$ -haemolysin in the toxin preparation, which presumably lysed these cells) it was decided to use mouse leucocytes. These cells were obtained from the peritoneal cavity of mice (weight 25—30 g) by injecting them intraperitoneally in the late afternoon with a sterile 2% starch solution in saline as irritant. The following morning the same mice were injected intraperitoneally with 4 to 5 ml sterile saline and about a half hour later an open Pasteur pipette was inserted into the peritoneal cavity of the living mouse and rotated slightly; in this way the material collected was almost pure exudate. The amount of exudate harvested per mouse varied greatly. There was no advantage in killing the mice. The exudates were harvested in a 1% solution of sodium citrate in saline pH = 7.2, centrifuged (3 min. at 1.500 rpm), and washed with saline several times. After the last centrifuging the sediment was again suspended in fresh saline and diluted until the suspension contained 5.000 leucocytes

per mm<sup>3</sup>. Exudate mixed with erythrocytes should be discarded as the red cells can cause undesirable side effects.

Ten successive dilutions mounting in fours were prepared from the toxin (1:4, 1:8, 1:12 etc.) and in volumes of 1 ml placed in tubes (1 cm O). 0.1 ml of the standard leucocyte suspension was added to each tube (including the control tube containing 1 ml of saline only). After shaking, the tubes were put in a waterbath (37°C.) for one hour and shaken every 15 minutes. Finally 0.1 ml of a 2% kaolin suspension in 1 N NaOH was added to each tube and the tubes shaken thoroughly. When lysis of the leucocytes occurs a homogeneous kaolin suspension ensues, when unchanged leucocytes are present they clot and form threads which become clearly visible owing to the sedimentation of kaolin on the cells. The tube with the highest dilution in which all the leucocytes were lysed was considered to be the endpoint. It is necessary to perform a control with the toxin dilution 1:4 without the addition of leucocytes in order to eliminate the possibility of the medium affecting the reaction.

TABLE 1.

| Strain | Toxins                   |                            |                                                                |                                              | Enzymes        |                  |                               |
|--------|--------------------------|----------------------------|----------------------------------------------------------------|----------------------------------------------|----------------|------------------|-------------------------------|
|        | Leuco-<br>cidin<br>titre | Haemoly-<br>sin            | Dermo-<br>necrotoxin<br>(seize in cm<br>of necrotic<br>lesion) | Lethal-<br>toxin<br>(death<br>after<br>min.) | Coagu-<br>lase | Fibri-<br>nolase | Hyaluro-<br>nidase<br>(titre) |
| 1      | 28                       | $\alpha$ and $\beta$       | $2.0 \times 7.0$                                               | 2                                            | +              | +                | 1.600                         |
| 2      | 8                        | $\alpha$ and $\beta$       | $1.7 \times 5.5$                                               | 40                                           | +              | +                | 800                           |
| 3      | 32                       | $\alpha$ and $\beta$       | Ø1.0                                                           | 15                                           | +              | +                | 800                           |
| 4      | < 4                      | $\alpha$                   | $2.0 \times 3.5$                                               | 270                                          | +              | +                | 1.600                         |
| 5      | 20                       | $\alpha$ and $\beta$       | $2.5 \times 5.0$                                               | 2                                            | +              | +                | 40.000                        |
| 6      | 20                       | $\alpha$ and $\beta$       | Ø1.0                                                           | 150                                          | +              | +                | 800                           |
| 7      | 24                       | $\alpha$ and $\beta$       | $1.5 \times 4.5$                                               | 10                                           | +              | +                | > 100.000                     |
| 8      | 24                       | trace $\alpha$ and $\beta$ | —                                                              | —                                            | +              | +                | 800                           |
| 9      | 12                       | trace $\alpha$ and $\beta$ | —                                                              | —                                            | +              | +                | 800                           |
| 10     | 8                        | $\alpha$ and $\beta$       | $1.7 \times 2.2$                                               | 330                                          | +              | +                | 1.600                         |
| 11     | 16                       | trace $\alpha$ and $\beta$ | —                                                              | —                                            | +              | +                | 800                           |
| 12     | 16                       | trace $\alpha$ and $\beta$ | —                                                              | —                                            | +              | +                | 800                           |

Strains 8 and 9 and strains 11 and 12 were isolated from the same patient at different periods in the course of the disease.

Table 1 gives a survey of the results. Only strain 4 produced such a small amount of leucocidin that even a 1:4 dilution did not cause complete lysis. A difference in reaction, however, was found between this strain and a number of apathogenic staphylococci used as control; the latter showed no lysis at all, while strain 4 demonstrated partial lysis in the dilution 1:4. It will be seen later this strain produces only  $\alpha$ -haemolysin, while all the other strains form  $\alpha$ - and  $\beta$ -lysin. According to VALENTINE (1936) the leucocidin of staphylococci measured by the technique of NEISSER-WECHSBERG would be identical with the  $\alpha$ -haemolysin; the leucocidin demonstrated by the method of PANTON and VALENTINE is different. FLAUM (1938) suggests that the leucocidin measured by the method of PANTON and VALENTINE may be identical with the  $\beta$ -haemolysin. The leucocidin demonstrated by the technique of TODD is not identical with the  $\beta$ -haemolysin, because strain 4 produces  $\alpha$ -lysin only. In this method, however, mice leucocytes have been used and no rabbit or human leucocytes such as is the case for the methods of NEISSER-WECHSBERG and of PANTON and VALENTINE.

When, working with TODD's method a correlation between leucocidin and haemolysins exists, it becomes more evident that the former toxin and  $\beta$ -haemolysin are identical. The identity of toxins, however, is a problem on its own.

### HAEMOLYSINS.

Although haemolysins have been found after leucocidin (KRAUS, 1900) they are better known and are the more important as a means of differentiating staphylococci. In 1901 NEISSER and WECHSBERG described a quantitative method, using filtrates from cultures of staphylococci, to estimate the concentration of haemolysins. By adding 0.5 % suspension of rabbit red cells to the filtrates they observed in a number of cases that the endpoint was higher when the mixture was allowed to stand in the refrigerator overnight, after one hour's incubation at 37°C. This phenomenon was again observed with a number of filtrates by WALBUM (1921), who called it "hot-cold" lysis.

BIGGER, BOLAND and O'MEARA (1927) made the following observations on this "hot-cold" haemolytic effect; during the "hot" period (2 hours at 37°C.) the haemolysins act on rabbit erythrocytes while only a trace of lysis is seen in the sheep red cells; in the following "cold" period (during night at 4°C.) lysis of rabbit erythrocytes practically ceases and lysis of sheep and human red cells is achieved. In 1935 GLENNY and STEVENS showed that both types of lysis were due to two different haemolysins which, however, were not formed by all staphylococcal strains. According to them the  $\alpha$ -lysin

acts on rabbit cells and partly on sheep cells at 37°C., it is dermonecrotic in guinea pigs and lethal in mice when inoculated intravenously in small quantities. The  $\beta$ -toxin gives a trace of lysis in sheep cells at 37°C., but it is haemolytic on subsequent cooling; it does not haemolyse rabbit cells, it is not dermonecrotic or lethal.

After this was known, the types of haemolysis of staphylococci on blood-agar plates have been investigated in more detail. Introductory investigations were performed by NAIDU and by BRYCE and ROUNTREE (1936), which have been confirmed and supplemented by CHRISTIE and GRAYDON (1941). On 5 % sheep blood-agar they distinguish 4 types of haemolysis:

- 1)  $\alpha$ -haemolysis: after 24 hours incubation a zone of haemolysis with a hazy margin is seen around the colony. No or very little change occurs when the plate is subsequently cooled.
- 2)  $\beta$ -haemolysis: after 24 hours incubation a sharply defined zone of discoloured cells is seen around the colony. On cooling the plates, a second zone of discoloration occurs, parallel to the outer edge of the first. No real haemolysis is seen, the effect of discoloration is thought to be due to the formation of methaemoglobin by the toxin. Staphylococcal strains producing both  $\alpha$ - and  $\beta$ -lysin are again subdivided into  $\alpha\beta$ -types and  $\alpha(\beta)$  types;
- 3)  $\alpha\beta$ -haemolysis: after 24 hours incubation a sharply demarcated zone of complete haemolysis is observed around the colony. On cooling a second zone of discoloration is usually formed round the first zone, which also shows a sharp margin.
- 4)  $\alpha(\beta)$ -haemolysis: after 24 hours incubation one sees a zone of complete haemolysis with a sharp margin surrounded by a zone of discoloration, again having a sharp edge. Subsequent cooling causes extension of both zones and frequently a third zone of discoloration occurs parallel to the outer edge of both the others.

Furthermore it appeared that  $\alpha$ -haemolysins are not found under anaerobic conditions, as distinct from the  $\beta$ -lysins, and that  $\alpha$ - and  $\beta$ -lysin together give rise to lysis on 2.5% human blood-agar plates, but independently they do not.

In our experiments the formation of haemolysin or haemolysins by each strain was determined by the tube technique with human, sheep and rabbit erythrocytes (titration); on sheep blood-agar plates both under aerobic and anaerobic conditions and on human blood-agar plates. The results of the plates were read after 24 hours' incubation at 37°C. and on subsequent cooling in the refrigerator overnight. Except for a minor modification in the composition of the medium, BURNET's technique (1930) was employed for the production of haemolysins.



Thus it was found that one strain (Nr. 4) formed  $\alpha$ -haemolysin only; the other strains formed as well as  $\alpha$ -lysin more or less  $\beta$ -lysin. Not all methods gave the same results. It was found that on sheep blood-agar plates, both under aerobic and anaerobic conditions, 3 strains did not form  $\beta$ -lysin, as distinct from the results of the titration-technique and on human blood-agar plates.

It appeared, moreover, that the strains 8, 9, 11 and 12, as will be shown later, did not form detectable amounts of dermo-necrotoxin or lethal toxin, but only produced a trace of  $\alpha$ -haemolysin as determined by the tube technique. This observation gives support to the suggestion of GLENNY and STEVENS (1935) that the  $\alpha$ -toxin would be identical with the dermo-necrotoxin and the lethal toxin of staphylococci.

No investigations into the formation of gamma or delta toxin have been carried out, because very little was known about these toxins at the time this investigation was done (1949).

#### DERMO-NECROTOXIN.

LINGELSHEIM (1900) and NEISSER and WECHSBERG (1901) observed that filtrates of cultures from a number of staphylococci, when inoculated subcutaneously into rabbits, caused large infiltrations at the site of injection and sometimes necrosis of the skin. No such reaction was observed in immunized animals nor when the toxic filtrate was previously neutralized by immune serum. PARKER (1924) investigated this reaction in more detail and found that filtrates containing this toxin gave rise to reactions of the skin within two hours, resulting in necrosis of the skin at the site of injection. Similar reactions have been observed in the skin of guinea pigs (TIMMERMAN, 1933). According to TIMMERMAN the latter animal was more suitable when using this skin reaction.

The medium used to obtain adequate production of this toxin was either 0.8% broth-agar (BURNET) or broth to which sodium formiate or sodium acetate was added to a final concentration of 0.5% (pH = 7.2). The addition of glucose to the broth appeared either to inhibit toxin production or to inactivate the toxin. An increased toxin production was achieved when the medium was incubated in an atmosphere containing 10–15% CO<sub>2</sub>. It appeared from comparative experiments that the highest concentrations of toxin were obtained after 6 to 8 days incubation at 37° C. Cultures of staphylococci were centrifuged for half an hour at 15,000 rpm,

the supernatant being used as toxin. Skin-tests were carried out in rabbits and guinea pigs simultaneously with these toxins; and it was found that guinea pigs appeared to be less sensitive than rabbits.

These investigations showed that the staphylococcal strains 8, 9, 11 and 12 do not produce dermo-necrotoxin.

#### LETHAL TOXIN.

This toxin was actually described first by NICOLLE and CÉSARI (1914); who found that a small amount of a Berkefeld filtrate from a culture of a staphylococcal strain isolated from a sheep, killed a rabbit or a guinea pig after intravenous inoculation within a few moments.

Highly toxic preparations were obtained by cultivating staphylococci on BURNET'S medium (0.8% broth-agar) by adding 1% proteose-peptone or 1% trypticase (B.B.L.) and by leaving out the glucose and phosphate buffer.

The inoculated plates were again incubated for one week at 37°C. in an atmosphere containing 10—20% CO<sub>2</sub>, subsequently centrifuged and the supernatant used as toxin preparation. Both rabbits and mice have been injected intravenously with the same results in both groups i.e. the latter animals died within 2 minutes to 5 hours after the injection of toxic preparations. The preparations which did not kill either mice or rabbits in amounts of 1 ml, were injected up to amounts of 10 ml into fresh rabbits without any effect being noted.

It appeared from these experiments that again the strains 8, 9, 11 and 12 did not produce lethal toxin. Rabbits inoculated intradermally with these centrifugates showed, with the exception of the above mentioned strains, a necrotic reaction of the skin.

Apart from toxins, staphylococci are able to form a variety of substrates of which it is not yet established whether they be toxic or provoke an antigenic action. *In vitro*, however, these substrates show properties which indicate that they can exert some influence upon the course of the infection. These substrates have a complex chemical structure and since their action in many ways shows a great similarity to those of certain enzymes or enzyme systems, they are generally considered as enzymes. They include substances

which cause the coagulation of plasma; which dissolve fibrin clots or prevent the clotting of plasma; and a substrate which increases the intercellular permeability of certain tissues.

#### COAGULASE.

Based upon the meritorious investigations of WILLIAMS and HARPER (1946), in which they compared eleven different techniques on the clotting of plasma by staphylo-coagulase with large numbers of staphylococcal strains, the coagulase tube test described by FISK (1940), giving the most reliable results, was chosen.

#### FIBRINOLASE-STAPHYLOKINASE.

Like streptococci, a number of staphylococcal strains can produce substances that cause lysis of fibrin clots. As far as is known, fibrinolase is non-antigenic, although LOOMIS, GEORGE and RYDER (1947) suppose a substance may be present in plasma capable of inhibiting the lysis. According to DYCKERHOFF and JACOBSEN fibrinolysin or fibrinolase (an esterase) exerts a proteolytic action. LACK (1948) demonstrated that the staphylofibrinolase has an action similar to streptokinase and he suggests the denomination staphylokinase. For both reasons it is preferable to call this substance fibrinolase or staphylokinase, instead of the old name fibrinolysin, especially as the latter might give rise to association with the toxins.

Intensive investigations have been carried out on fibrinolases produced by streptococci. The techniques applicable to streptococci, however, can not be used for staphylococci, and up to now no reliable method is available in demonstrating the production of fibrinolase by staphylococci. Analogous to the method of CHRISTIE and WILSON (1941) used for streptococci, i.e. broth agar with the addition of 12% oxalate plasma, is the technique which has been applied to staphylococci. Plates are poured with this turbid medium and inoculated with staphylococci.

Based upon the following considerations the clearing of the agar round the colonies indicates the formation of fibrinolase by staphylococci; 1° because the same clearance is observed with haemolytic streptococci, and 2° because by substituting a fibrin solution in saline for plasma in the medium, the same results are observed.

No complete correlation has been found between the production of coagulase and fibrinolase. Whether significance can be attached to the formation of fibrinolase as a means of differentiating the pathogenic from the non-pathogenic staphylococci has not yet been established, since it has been found that many animal-pathogenic strains do not clear the plasma-agar.

The twelve strains investigated here, appeared to produce coagulase and caused clearance of the plasma-agar plates.

## HYALURONIDASE.

In experiments on the production of dermo-necrotoxin by a number of staphylococci PARKER (1924) already has described that the bleb (which is the direct result of the intradermal injection of a small amount of filtrate from a staphylococcal culture), flattened nearly instantaneously in a number of cases as distinct from the control (sterile broth). To this phenomenon no further attention was paid until DURAN-REYNALS (1933) showed that a number of bacteria may form a substance or substances, increasing the permeability of some tissues (spreading factor). He inoculated rabbits intradermally with filtrates of staphylococcal cultures, especially of those isolated from pathologic material. It appeared that the majority of these strains showed a close correlation between the production of spreading factor and the cultural characteristics in connection with their pathogenicity.

The determination of hyaluronidase was possible only for biochemists until Mc CLEAN'S (1943) observation that the first stage during the enzymic hydrolysis of hyaluronic acid was the destruction of the power of the substrate to clot with acetic acid (mucin-clot prevention test). This made it possible to demonstrate hyaluronidase by an indirect reaction with rather simple expedients. The only disadvantage of this method lay in that a pure preparation of hyaluronic acid was necessary, which was not required for the A.C.R.A. test (Congo-Red-in-Acid Alcohol) as described by BURNET (1948).

Several detailed studies on the kinetics, production and other properties of hyaluronidase have been made. Enzyme production on a simplified medium has been obtained, though some organisms require complex factors, such as the addition of hyaluronic acid to the medium. A medium containing casein hydrolysate, amino acids, biotine and salts is recommended when dealing with the staphylococcal group. Although glucose promotes the growth of staphylococci, it affects the pH unfavourably and ROGERS (1945) added 2½% sodium glycerophosphate in stead of glucose. With two staphylococcal strains he found enzyme titres of 1:100 and 1:3,200 by the mucin-clot prevention test.

For the assay of hyaluronic acid BURNET (1948) employed one of the properties of this substrate, i.e. the one which is coagulated by acid alcohol. When this mucopolysaccharide is dropped into acid alcohol a solid film is formed along the contact surfaces of both substances and the drop becomes spherical or annular depending upon the mucin concentration. If no mucin is present the drop spreads directly and leaves a cloudy patch behind on the bottom of the dish. Pure hyaluronic acid does not give such a well-defined pattern, but synovial fluid, which contains a high concentration of mucin, does so. BURNET obtained human synovial fluid by injecting 10 ml sterile saline into the knee-joint of a corpse followed by the extraction of as much fluid as possible. Before the reaction was performed the synovial fluid was diluted 10—20 times and a small amount of congo red was added.

Synovial fluid from cows was used as substrate in our experiments. Under sterile conditions and using a large syringe this fluid was



sucked from the opened knee-joint of a freshly slaughtered cow. When stored in a refrigerator, this sterile fluid can be used for several months. The highest dilution showing hyaluronidase activity, i.e. the formation of a ring ("doughnut") when dropped in acid alcohol, was determined. The borate buffer of BURNET and STONE (1947) with the addition of calcium chloride <sup>1)</sup> was used as diluant. The hydrolysis of hyaluronic acid by the enzyme hyaluronidase ensues more rapidly when calcium ions are added to the mixture.

Successive dilutions mounting in tens were prepared from the synovial fluid with the buffer. To each tube one drop of a 1% watery congo red solution was added per 0.5 ml of dilution. BURNET advised 2 drops of a 0.1% solution of congo red, but with 1 drop of a 1% solution a sharper outline of the clot was obtained in acid alcohol. On the other hand, the addition of too much congo red interfered with the sensitivity of the reaction. A few drops from each tube were dropped into a thin layer (0.5 cm) of a 1% solution of concentrated HCl in 70% alcohol, in a flat bottomed Petri dish placed preferably on a white background. By adding congo red to the mucin the drop is stained dark blue by the acid alcohol solution. In the lower dilutions (1 : 10, 1 : 20) of the synovial fluid fading of the drop is often observed; in somewhat higher dilutions (1 : 30—1 : 50), however, a dark blue, lenticular clot is formed; and in still higher dilutions (1 : 50—1 : 80) the drop forms a dark blue ring with well-defined inner and outer margins. At a certain dilution (1 : 60 to 1 : 120) the outer margin of the ring will spread directly after the drop has fallen into the acid alcohol, although the inner margin remains well-defined and intact. Higher dilutions give spreading of the whole drop. The highest dilution giving a well-defined ring in the acid alcohol was chosen as endpoint; when this appeared to be a dilution of 1 : 80 of the synovial fluid, then this should not be diluted to more than 1 : 40 for the A.C.R.A. test itself (standard mucine dilution), because the addition of the same amount of culture filtrate to this dilution will bring the mucin concentration down to 1 : 80. When too high mucin concentrations are used the action of the enzyme is less intensive.

The enzyme from the different staphylococcal strains was ob-

---

<sup>1)</sup> CaCl<sub>2</sub> 1.0 g; NaCl 9.0 g; H<sub>3</sub>PO<sub>4</sub> 1.203 g; Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> · 10 H<sub>2</sub>O 0.052 g; Aq.dest. ad 1000. pH = 7.0.



tained by inoculating and incubating the following medium: Trypticase 2.0 g, Dextrose 0.25 g, Sodium glycerophosphate, Aqua destillata ad 100 ml, for 24 hours. Subsequently the medium was centrifuged for 1 hour at 15,000 rpm, and the supernatant harvested.

Serial twofold dilutions (1 : 100, 1 : 200, 1 : 400 etc.) of the enzyme (staphylococcal culture-centrifugate), were made up in calcium borate buffer in volumes of 0.25 ml. Each tube, provided with 0.25 ml of a standard mucin dilution, was placed into a waterbath and left for 1 hour at 37°C. Subsequently to each tube 1 drop of a 1% congo red solution was added.

Two controls were run in addition to the foregoing, first: 0.25 ml of standard mucin dilution and 0.25 ml of calcium borate buffer, which must give a well-defined dark blue ring when dropped in acid alcohol, after being kept for 1 hour at 37°C.; and second: a one by one dilution of the culture centrifugate with borate buffer, giving a spreading out of the drop in acid alcohol.

Readings were taken as described above in the titration of the mucin (synovial fluid); that dilution of the enzyme which gave a diffuse outer margin and an intact, well-defined inner margin in acid alcohol was considered to be the endpoint of hyaluronidase activity.

Table 1 gives a survey of the titres found in those dilutions from the culture filtrates which showed hyaluronidase activity.

From these titres it appeared that all strains produce hyaluronidase and that the only albus-strain (7) forms a particularly large amount of this enzyme. These reactions were repeated several months later with the same centrifugates and also with freshly prepared centrifugates. The results were identical. Control tests performed with coagulase-negative staphylococci failed to show enzyme activity even when undiluted.

It lies beyond the scope of this work to enter into details concerning the capacity of the production of hyaluronidase and its connection with the pathogenicity of staphylococci. Because no biochemical reaction for the determination of the pathogenicity of staphylococcal strains has been found to be reliable, the A.C.R.A. test, which can be easily applied in routine work, opens new possibilities for extensive research in this field.

## DISCUSSION AND CONCLUSIONS.

A series of experiments were carried out to obtain some idea of the ways in which staphylococci, isolated from sputum of patients suffering from a secondary staphylococcal pneumonia during influenza or from the lungs after autopsy, can injure the tissues of their hosts by certain of their products (toxins, enzymes) and exert a deleterious effect on the progress of the primary influenza viral infection.

It is known, that some strains of staphylococci can form toxins and other active constituents or products that are not markedly toxic in themselves, but which act upon tissues, cells or fluids in such a way that they may influence the course of the bacterial infection, or at least contribute to the virulence or pathogenicity of the microorganism. It is generally accepted that pathogenic strains of staphylococci produce a larger number of toxins, enzymic and nontoxic substances than the so-called saprophytic strains. Without a doubt all these substrates play an active part in the genesis of the infection, but it has been impossible until now to demonstrate their action owing to morphological changes in tissue sections, and we have to rely on *in vitro* reactions to demonstrate their presence.

Of the total 12 strains, among which 2 pairs appeared to be identical, eleven belonged to the species *M. pyogenes* var. *aureus* and one to the var. *albus* (strain 7). All strains showed a great metabolic activity, which gives a good indication of their pathogenicity; in addition to this all strains produced lipase and coagulase, exerted fibrinolytic activity and produced hyaluronidase. The only *albus*-strain appeared to produce hyaluronidase, in very large quantities when compared with the *aureus*-strains.

Eleven out of twelve strains, including the *albus*-strain, formed leucocidin according to TODD's method. Eleven strains produced  $\alpha$ -haemolysin and  $\beta$ -lysin, although in varying amounts; one strain produced  $\alpha$ -lysin only and, as it appeared, the same strain which produced no detectable amount of leucocidin. This observation supports the suggestion of FLAUM (1938) that the leucocidin demonstrated by the method of PANTON and VALENTINE and consequently probably according to TODD's method as well, may be identical to the  $\beta$ -haemolysin.

Only eight out of twelve strains, including the *albus* strain, produced a toxin when injected into the skin of rabbits, gave rise to necrosis and, when injected intravenously into the rabbit or mouse caused acute and fatal toxæmia. On comparing the results it appeared that the four strains which did not produce dermo-necrotoxin or lethal toxin only produced a trace of  $\alpha$ -haemolysin, which supports the suggestion of GLENNY and STEVENS (1935) that  $\alpha$ -haemolysin might be identical to both the dermo-necrotoxin and lethal toxin. That 4 out of 12 strains did not form detectable amounts of dermo-necrotoxin and lethal toxin indicates that this reaction, in the judgment of the pathogenicity of staphylococci is of relative value only: the more so as it appeared that these strains gave pathological and histological pictures in the lungs of mice similar to the other staphylococcal strains (VAN TONGEREN, 1951).

### References.

- BIGGER, J. W., BOLAND, C. R. and O'MEARA, R. A. Q. 1927. *J. Path. Bact.* **30**, 271.
- BRYCE, L. M. and ROUNTREE, P. M. 1936. *J. Path. Bact.* **43**, 173.
- BURNET, F. M. 1930. *J. Path. Bact.* **33**, 1.
- BURNET, F. M. and STONE, J. D. 1947. *Austr. J. exp. Biol. Med. Sc.* **25**, 219.
- BURNET, F. M. 1948. *Austr. J. exp. Biol. Med. Sc.* **26**, 371, 381, 389.
- CHRISTIE, R. and GRAYDON, J. J. 1941. *Austr. J. exp. Biol. Med. Sc.* **19**, 9.
- CHRISTIE, R. and WILSON, H. 1941. *Austr. J. exp. Biol. Med. Sc.* **19**, 329.
- MC CLEAN, D. 1936. *J. Path. Bact.* **42**, 477.
- MC CLEAN, D. 1943. *Biochem. J.* **37**, 169.
- DURAN REYNALS, F. 1933. *J. exp. Med.* **58**, 161.
- DYKERHOFF, H. and JACOB, O. 1900. *Biochem. Z.* **317**, 72.
- EYKMAN, C. 1901. *Z. f. Bakt. Abt. I*, **29**, 841.
- FISK, A. 1940. *Brit. J. exp. Path.* **21**, 311.
- FLAUM, A. 1938. *Acta Path. Microbiol. Scand. Suppl.* **35**, 1.
- GLENNY, A. T. and STEVENS, M. F. 1935. *J. Path. Bact.* **40**, 201.
- KRAUS, R. 1900. *Wien. Klin. Wschr.* **13**, 49.
- VON LINGELSHEIM, W. 1900. *Aetiologie u. Therap. d. Staph. Inf.* Berlin-Wien.
- LOOMIS, E. C., GEORGE, E. and RYDER, A. 1947. *Ref. Arch. Biochem.* **12**, 1.
- NEISSER, M. und WECHSBERG, F. 1901. *Z. Hyg.* **36**, 299.
- NICOLLE, M. and CESARI, E. 1914. *Ann. Inst. Past.* **28**, 219.
- PANTON, P. N. and VALENTINE, F. C. O. 1932. *Lancet* **222**, 506.
- PARKER, J. T. 1924. *J. exp. Med.* **40**, 761.
- ROGERS, H. J. 1945. *Biochem. J.* **39**, 435.
- TIMMERMAN, W. A. 1933. *Ned. Tijdschr. Hyg. Microbiol. Serol.* **7**, 478.
- TODD, E. W. 1942. *Brit. J. exp. Path.* **23**, 136.

- VAN TONGEREN, H. A. E. 1951. An experimental study on influenza and secondary staphylococcal pneumonia in mice. Thesis Utrecht.
- VALENTINE, F. C. O. 1936. *Lancet* **230**, 526.
- WALBUM, L. E. 1921. *C. R. Soc. Biol.* **85**, 1205.
- WILLIAMS, R. E. O. and HARPER, G. J. 1946. *Brit. J. exp. Path.* **27**, 72.
-

(From the Department of Bacteriology and Experimental Pathology of the Netherlands' Institute for Preventive Medicine, Leiden).

## THE ISOLATION AND SOME CHARACTERISTICS OF BACTERIOPHAGES FROM STAPHYLOCOCCI PRESENT IN CASES OF SECONDARY PNEUMONIA FOLLOWING INFLUENZA

by

**H. A. E. VAN TONGEREN**

(Received April 18, 1953).

An attempt was made to gain some insight into the epidemiologic relationship of the staphylococcal strains mentioned in a previous paper (VAN TONGEREN, 1953), which were isolated within a short period of time both from different hospitals in several towns and from a single hospital, during the same influenza epidemic, by means of phagetyping.

Serological reactions for the differentiation of staphylococci being of minor significance, the object was to isolate bacteriophages from the staphylococcal strains. Staphylococcal phages have been readily isolated from lysogenic strains using the cross-culture method of FISK (1942). Most authors have described the isolation of these phages from pathogenic strains of human origin (WILSON and ATKINSON, 1945; ROUNTREE, 1947; WILLIAMS SMITH, 1948) and once a number of type 42 D strains of bovine origin was investigated by the cross-culture method (WILLIAMS SMITH, 1948).

As we did not possess the standard typing phages of WILSON and ATKINSON (1945), an investigation was undertaken to determine how many phages could be isolated from these strains by the cross-culture method, and to study the cross reactions and some biological characteristics. Furthermore an attempt was made to find in how far an "epidemic strain" of staphylococci could be demonstrated, since some workers (e.g. FINLAND, PETERSON and STRAUSS, 1942) have suggested the existence of an epidemiological relationship between staphylococci isolated from secondary pneumonia during epidemics of influenza.



## MATERIALS AND METHODS.

Twelve staphylococcal strains have been investigated for the presence of phages.

## Isolation of the phages.

The medium used for all phage work comprised the following substances: Trypticase (B.B.L.) 2.0 g; glucose 0.1 g; sodium chloride 0.5 g; agar 1.5 g; Aq. dest. 100 ml. pH = 7.2. The technique used for isolation and purification of the phages was the original streak method of FISK (1942) i.e. the basal strain was streaked over the agar and after drying in the incubator at 37°C. for half an hour a droplet of each of the other eleven strains was dropped separately on the streaks by a Pasteur pipette. In this way each of the strains was used as basal strain once, for a lytic action was occasionally observed in one combination only. The plates were incubated at 37°C. for 6 to 8 hours then left 24 to 48 hours at room temperature to obtain larger plaques.

Hartley broth (a mixture of equal amounts of meat-broth and saline, pH = 7.6) was used for suspending the phages collected from the agar. Suspending in distilled water, as Fisk advised, kills the phages very easily, and as the use of sterile saline for filtration of phages to separate them from the cocci, appeared to be disadvantageous owing to absorption of many phages in the filter (as with other viruses: ELFORD, 1937).

The lysogenic and lytic strains were determined directly after the first isolation, by dropping the phage on both strains from which it was derived. When after several passages on the agar confluent lysis by the phage of the propagating (lytic) strain was obtained, the agar was collected and washed in Hartley broth. Phage preparations were then filtered through Seitz-EK filters, but in a number of cases the Chamberland L 2 filter candle had to be used. The phages were designated by the method of WILSON and ATKINSON, the numerator indicating the organism from which they were derived (lysogenic strain) and the denominator indicating the strain on which they were propagated (lytic strain). In one case both staphylococcal strains appeared to be lysogenic i.e. both contained a phage causing lysis of the other strain [phage 1/8 (1/9) and phage 8/1 (9/1)]. After filtering, each filtrate was tested for bacterial sterility and for its lytic action on the propagating strain.

## Titration of phages.

Titration of phage suspensions was carried out by the method of CRAIGIE and YEN (1938) used for the typing of *S. typhi* with Vi-phage, adapted to the temperature and time of incubation of staphylococcal phages. The critical test dilution (C.T.D.) was recorded as the highest phage dilution just giving confluent lysis in the propagating (lytic) strain. This dilution was used in the titration tests throughout. Pure phage suspensions were stored in the refrigerator at 4° C. and could be used for several weeks without fall in titre.

## RESULTS.

The cross-culture test on first passage showed that a number of strains were susceptible; consequently there were lysogenic strains. In this way a total of 22 phages were isolated and propagated, of which undoubtedly a number would appear to be identical. By comparison and selection the phages appeared to be carried by the strains 1, 2, 8 and 9, and 11 and 12 (lysogenic).

Some phages, however, gave aberrant reactions and these were: phage 2/8 propagated on the susceptible strain 2, i.e. phage 2/8/2, phage 2/9 propagated on the susceptible strain 2, i.e. phage 2/9/2, phage 11/1 propagated on the susceptible strain 11, i.e. phage 11/1/11 and phage 12/1 propagated on the susceptible strain 12, i.e. phage 12/1/12.

We did not succeed in isolating the phages 8/2, 9/2, 1/11 and 1/12 directly. The phages isolated from the first crossing of strain 8 on strain 2, of strain 9 on strain 2, of strain 1 on strain 11 and of strain 1 on strain 12, disappeared by subculturing on the original basal strain (being the strains 2, 11 and 12 respectively). This phenomenon was observed repeatedly with the same strains.

After isolation and purification the phages were propagated on the susceptible strains and diluted to the C.T.D. for typing.

Another remarkable observation presented itself on filtrating the phages to separate them from the propagating strain to obtain pure phage preparations. A number of the phage suspensions, after having passed through the Seitz EK filter, either failed to produce lysis or produced only some discrete plaques on the lytic strain, although before filtration confluent lysis was observed on the same strain. Similar finding were present after filtration through Chamberland (L 3) candles.

When, however, Chamberland L 2 candles were used these phages passed the filter readily, while the cocci were retained. As far as is known GOLDSMITH (1937) made a similar observation with three staphylococcal phages which appeared to be retained by a Chamberland L 3 filter candle.

According to GOLDSMITH these differences in the filterability of phages did not depend either upon the age of the filtrates or on the temperature at which they were filtrated or stored. Neither contact with homologous cultures nor mixing them with phage strains which would pass the filters made any difference.

The most plausible explanation, however, that staphylococcal phages might be of different sizes was not made. According to the pore size of the filters they vary from 35 to 100  $m\mu$ . Electron microscope studies by VAN ROOYEN revealed that the length of staphylococcal phages never exceeded 300  $m\mu$  and their breadth is 50  $m\mu$ .

All phages derived from the lysogenic strains 1, 8 and 9 did not pass the Seitz EK filter and Chamberland L 3 filter candles. The other phages originating from the lysogenic strains 2, 11 and 12, however, could be readily filtered.

The result of the titration test with all phages showed that, as might have been expected, a number appeared to be identical *viz.*, strains 8 and 9, and 11 and 12 respectively.

A survey of the titrations of all phages on the strains are recorded in table 1.

TABLE 1.

| Phage   | Staphylococcal strains |    |   |   |   |   |   |    |    |    |     |     |
|---------|------------------------|----|---|---|---|---|---|----|----|----|-----|-----|
|         | 1                      | 2  | 3 | 4 | 5 | 6 | 7 | 8  | 9  | 10 | 11  | 12  |
| 2/8/2   | CL                     | CL | — | — | — | — | — | —  | —  | —  | —   | —   |
| 2/9/2   | CL                     | CL | — | — | — | — | — | —  | —  | —  | —   | —   |
| 8/1     | CL                     | —  | — | — | — | — | — | —  | —  | —  | —   | —   |
| 9/1     | CL                     | —  | — | — | — | — | — | —  | —  | —  | —   | —   |
| 2/8     | CL                     | —  | — | — | + | — | — | CL | CL | —  | ++  | ++  |
| 2/9     | CL                     | —  | — | — | + | — | — | CL | CL | —  | ++  | ++  |
| 2/1     | CL                     | —  | — | — | — | — | — | CL | CL | —  | —   | —   |
| 1/8     | ++                     | —  | — | — | — | — | — | CL | CL | —  | +++ | +++ |
| 1/9     | ++                     | —  | — | — | — | — | — | CL | CL | —  | +++ | +++ |
| 11/1    | CL                     | —  | — | — | — | — | — | +  | +  | —  | —   | —   |
| 12/1    | CL                     | —  | — | — | — | — | — | +  | +  | —  | —   | —   |
| 11/1/11 | —                      | —  | — | — | — | — | — | CL | CL | —  | CL  | CL  |
| 12/1/12 | —                      | —  | — | — | — | — | — | CL | CL | —  | CL  | CL  |

CL = confluent lysis; + to +++ = partial lysis.

As phages 1/8/1, 1/9/1 etc., were identical with the phages 1/8, 1/9 etc. they have been omitted from this table.

On dropping strain 2 on strains 8 and 9 (basal strains) a phage was isolated which could only be propagated on strain 2, whereas on dropping strains 8 and 9 on strain 2 (basal strain) a few plaques have been observed although with subcultivation on strain 2 this phage disappeared. Of phages 2/8/2 and 2/9/2, strains 8 and 9 were lysogenic and strain 2 lytic; strains 8 and 9 therefore contain a phage giving lysis of strain 1 (phages 8/1 and 9/1) only and phages (2/8/2 and 2/9/2) giving lysis of both strains 1 and 2. Two possibilities may account for this:

- a. that strains 8 and 9 contain 2 different phages or
- b. that both phages are identical, but by propagating on, and thus adapting to strain 2, they caused lysis of both strains 1 and 2. The fact that direct crossing of the strains 8 and 9 on strain 2 gave no phage which could be constantly propagated on strain 2, is in favour of the adaptation.

Nearly the same conclusions can be drawn on closer inspection of the action of phages 2/8, 2/9 and 2/1. Of these phages strain 2 is the lysogenic one. When this phage is propagated on the susceptible strain 1, then only the strains 1, 8 and 9 give confluent lysis with the C.T.D. This phage, however, propagated on strains 8 and 9, also causes confluent lysis of the strains 1, 8 and 9, and trace reactions are observed of the strains 5, 11 and 12. These trace reactions can be explained simply by accepting that propagation of the phage originating from strain 2 on strains 8 and 9, causes trace reactions on other strains by adaptation to strains 8 and 9.

A rather peculiar picture is seen after typing with the phages 1/8 and 1/9. These phages cause confluent lysis of their propagating strains and in addition a partial lysis of strains 11 and 12 is seen; a trace reaction is also observed of strain 1. This would be incompatible with the generally accepted conception that the phage of the lysogenic strain can only lyse the lytic strain and never the lysogenic one.

Table 1 shows that by crossing of strains 8 and 9 on strain 1 a phage was isolated which caused lysis of strain 1 only. The only way in which partial lysis of strain 1 by phages 1/8 and 1/9 can be explained is that a mixture of two phages existed and the lytic power of one phage (8/1) was suppressed by propagating it on strains 8 and 9.

Trace reactions were also observed of phages 11/1 and 12/1 on strains 8 and 9.

Superficial observation of the lytic action of phages 11/1/11 and 12/1/12 indicates that strains 8 and 9 are identical with strains 11 and 12; evidence to the contrary is present, however, in that phage 2/1 gives confluent lysis of strains 8 and 9 but no lysis of strains 11 and 12, and that a phage was isolated by crossing strain 2 with strains 8 and 9 but not with strains 11 and 12.

Although only 10 staphylococcal strains have been actually tested by phage, no epidemic strain could be found either by examination of biochemical properties, toxin and enzyme formation or by phagotyping, irrespective of the fact that several strains were isolated in one single hospital and the others in different parts of this country during the same epidemic of influenza.

### S u m m a r y.

No epidemic strain assumed by some investigators to be present during influenzal epidemics could be demonstrated by means of phage typing; it was observed furthermore, that phages isolated from different staphylococcal strains show a variation in size as measured by filtration, which enables them to be divided into groups.

### R e f e r e n c e s.

- CRAIGIE, J. and YEN, C. H. 1938. *Can. Publ. Health J.* **29**, 338, 484.  
ELFORD, W. J. 1936. *Brit. J. exp. Path.* **17**, 399.  
FINLAND, M., PETERSON, O. L. and STRAUSS, E. 1942. *Arch. Int. Med.* **70**, 183.  
FISK, R. T. 1942. *J. Inf. Dis.* **71**, 153, 161.  
GOLDSMITH, N. R. 1937. *J. Bact.* **33**, 495.  
VAN ROOYEN, C. E. and RHODES, A. I. 1948. *Virus diseases of man*. New York.  
VAN TONGEREN, H. A. E. 1951. An experimental study on influenza and secondary staphylococcal pneumonia in mice. Thesis Utrecht.  
VAN TONGEREN, H. A. E. 1953. *Antonie van Leeuwenhoek* **20**, 65.  
SMITH, H. W. 1948. *J. Comp. Path. Ther.* **58**, 180; *J. Hyg. Camb.* **46**, 74, 82.  
WILSON, G. S. and ATKINSON, J. D. 1945. *Lancet* **248**, 647.



(Department of Bacteriology and the Live Stock Sanitary Service Laboratory, University of Maryland, College Park, Maryland).

## THE SPECIES OF *ERYSIPELOTHRIX*

by

GEORGE C. LANGFORD, Jr.<sup>1</sup> and P. ARNE HANSEN

(Received May 9, 1953).

The sixth edition of BERGEY's Manual (1), published in 1948 follows ROSENBAACH (12) in the recognition of three species in the genus *Erysipelothrix* Rosenbach: *E. rhusiopathiae*, *E. muriseptica*, and *E. erysipeloidis*<sup>2</sup>). However, the descriptions of the three species appear to indicate no real morphological, cultural, serological, or biochemical differences. The separation into species seems to be based entirely upon the animals originally infected. Ever since RICKMANN (2) first published his work on swine erysipelas, erysipeloid, and mouse septicemia in 1909, many workers have thought that the division of the genus into several species was unwarranted and that all forms could legitimately be included in one species.

Organisms causing mouse septicemia, swine erysipelas, and human erysipeloid have, at various times, been placed in the following genera: *Bacillus* Trevisan (3), *Pasteurella* De Toni and Trevisan (4), *Babesia* Trevisan (5), *Oospora* Savageau and Radais in BRUMPT (6), *Bacterium* Migula (7), *Streptothrix* Kruse (8), *Actinomyces* Lachner-Sandoval (9), *Mycobacterium* Chester (10), *Discomyces* Gedoelst (11), *Erysipelothrix* Rosenbach (12), and *Nocardia* Castellani and Chalmers (13). This is not surprising since this organism is highly pleomorphic and can assume forms ranging from coccoid rods to long tangled chains of filamentous structures. Granules, which some of the early workers considered to be spores, may or may not be present.

<sup>1</sup>) Present address, Department of Bacteriology, University of Florida, Gainesville, Florida.

<sup>2</sup>) The names actually used by ROSENBAACH were respectively: *Erysipelothrix porci*, *E. murisepticus* (sic), and *E. Erysipeloides* (sic).

The first valid generic name for this group of organisms seems to be *Erysipelothrix* Rosenbach, which was recommended in a report of the "Committee of the Society of American Bacteriologists on characterization and classification of bacterial types" WINSLOW *et al.* (14). They designated *E. rhusiopathiae* as the type species. Several older generic names exist but of these only *Babesia* and *Discomyces* need any comment in a discussion of priority. *Babesia* proposed by TREVISAN (5) contains a description of two species of elongated cocci occurring in pairs, *Babesia xanthopyrethica*, supposedly the cause of yellow fever, and *Babesia erysipeloidis*. In the same paper the swine erysipelas organism was placed in the genus *Bacillus*. It appears that, quite apart from the probable invalidity of the name *Babesia* as such, its use for an organism of the erysipeloithrix group was in error. *Discomyces* may be considered as a synonym of *Actinomyces*, see BUCHANAN (15).

Not only has the generic name been altered frequently, but the specific epithets have been subject to change. The number of species as well as the names used for them have varied with the authors. Some workers, anxious to subdivide the group into several species, have attempted to use host relationships or minute variations in cultural appearance as differential characters. Others have disclaimed the validity of such subtle distinctions and have placed the varieties within one species, ascribing the differences to variability of the organism. Thus SCHÜTZ (16), SMITH (17), LEHMANN and NEUMANN (18), CHESTER (10,19), and PRETTNER (20) considered this group of organisms to be identical or nearly so no matter what the animal source. ROSENBACH (12) however, attached considerable importance to the cultural and morphological appearance of the three strains in gelatin and on solid media and on this basis, he separated the group into three species. RICKMANN (2) pointed out that ROSENBACH had studied one single culture from a case of erysipeloid and, that his results were therefore liable to error. By studying ROSENBACH's culture from erysipeloid, and by examining more than 100 other strains isolated from rotlauf cadavers and cases of mouse septicemia, RICKMANN could find no real morphological difference between them. Since the immune and agglutination reactions were also about the same for all three groups of organisms he considered them to be identical.

TENBROECK (21), TEICHMANN (22), ZIBERT (23), ZOSEL (24), KLAUDER (25), HARKINS (26), KONDO and SUGIMURA (27), KOHL (28),

and others have indicated that they consider the causative organisms of swine erysipelas, human erysipeloid and mouse septicemia as well as some which can be isolated from a number of other animals, to be identical from a morphological, cultural, physiological and serological standpoint. More recently, LANGFORD (29) arrived at the same conclusion in a study of the identity of these organisms. Lately BYRNE *et al.* (30) have studied the morphological, cultural and biochemical characteristics of 141 strains of *Erysipelothrix rhusiopathiae*. Their findings agree in all major points with the cultural characters described below.

#### EXPERIMENTAL.

In the present investigation the cultural characters of 50 strains of *Erysipelothrix* were studied in an attempt to differentiate between varieties which might exist. Strains designated as *Erysipelothrix rhusiopathiae*, *E. muriseptica*, or simply as *Erysipelothrix* sp. were collected from widely separated sources. Cultures were obtained from Alabama, North Carolina, Iowa, Maryland, Minnesota, New York, Pennsylvania and Texas; and from Australia, Denmark, England and France. They were isolated from pigs, turkeys, ducks, lambs, mice and an African jacana (in the London Zoo). In addition, several strains were secured which had been passed through pigeons, hamsters and turkeys. Two strains were obtained from hog cholera virus vaccine. No strain of *E. erysipeloidis* was available for study.

The following mixture was found very useful as a base for the preparation of liquid carbohydrate media, solid media, and differential media: Trypticase, 4%; yeast extract, 1%; adjusted to pH  $7.8 \pm 0.1$ . The carbohydrates and other compounds to be fermented were added in 1% concentration and the media Seitz filtered. After incubation for 1 week at 33°C. the pH was determined directly in the culture by the glass electrode. All of the strains were remarkably similar from standpoints of morphology, cultural characteristics, and physiological activity. They assumed forms ranging from short coccoid rods to very long thin filamentous structures. Early cultures on favorable media were consistently gram positive. Older cultures frequently failed to retain the gram stain and the occurrence of large granules was not uncommon. Isolated colonies on trypticase-yeast extract agar attained a diameter of 1 to 1.5 mm, were transparent by transmitted light, and showed a slight bluish sheen by

reflected light. Colonies on tellurite media were similar in size but were jet black in color. A typical "test tube brush" type of growth was generally found in gelatin stabs, but, seven strains failed to produce this characteristic growth. The organisms developed well in media which had an initial pH of 7.3 to 9.0, and were inhibited usually below pH 6.4. All strains regularly produced acid in media containing fructose, glucose, galactose, and lactose. Reactions on other carbohydrates and polyalcohols were variable depending upon the medium employed. All strains showed considerable tolerance to sodium chloride in concentrations up to 7%, phenol in concentrations to 0.2% and potassium tellurite in concentrations to 0.05%. The minor variations which were found among the organisms studied, did not appear to be sufficiently significant to warrant species differentiation and could not be related to the present separation into three species.

#### DISCUSSION.

The inconvenience of having 3 species in the genus *Erysipelothrix* without having any diagnostic means by which to separate them is obvious. To separate bacteria merely on the basis of source is unscientific and should be abandoned. We recommend that they be included in one species, which, according to the International Code of Nomenclature (31), Rule 19, should be given the oldest specific name. It appears that the epithet *insidiosus* applied by TREVISAN (3) in 1885 is valid and has priority. He clearly indicated that the organism was the causative agent of mouse septicemia, although he erroneously described it as a spore-forming rod. The mention of "spores" in older works can not be considered equivalent to the finding of true endospores in the present meaning of this term. TREVISAN quotes the work of ZOPF (32, p. 88) whose description is in part based on the paper by KOCH 1878 (33). These detailed descriptions leave no doubt that the organisms investigated were typical *Erysipelothrix*. When revising the generic name the specific epithet must be changed to conform in gender. Hence *Bacillus insidiosus* Trevisan must be changed to *Erysipelothrix insidiosa* (Trevisan) comb. nov.

The authors are gratefully acknowledging their indebtedness to their colleagues in other laboratories who kindly furnished cultures for this investigation and regret that space does not permit the mention of each individual worker.

## Summary.

The three species *Erysipelothrix rhusiopathiae*, *E. erysipeloidis*, and *E. muriseptica* should be united into one, to which the oldest valid specific name should be applied. The correct name is *Erysipelothrix insidiosa*.

## Literature.

1. BREED, R. S., MURRAY, E. G. D., and HITCHENS, A. P. 1948. Bergey's Manual of Determinative Bacteriology. 6th Ed. Williams and Wilkins, Baltimore.
2. RICKMANN, W. 1909. Z. Hyg. **64**, 362.
3. TREVISAN, V. 1885. Atti della Acad. Fisio-Medico-Statistica, Milano, Ser. 4, **3**, 92.
4. DE TONI, J. B. and TREVISAN, V. 1889. In SACCARDO, P. A., Sylloge Fungorum **8**, 995.
5. TREVISAN, V. 1889. I Generi e le specie delle Batteriacee. 13, L. Zanaboni e Gabuzzi, Milano.
6. BRUMPT, E. 1927. Précis de Parasitologie. 4th Ed. 1201, Masson et Cie., Paris.
7. MIGULA, W. 1895. In ENGLER and PRANTL, Die Natürlichen Pflanzenfamilien. **1**, 1a, **24**, Leipzig.
8. KRUSE, W. 1896. In FLÜGGE, C., Die Mikroorganismen. 3rd Ed., **2**, 61, F. C. W. Vogel, Leipzig.
9. LACHNER-SANDOVAL, V. 1898. Ueber Strahlenpilze, Strassburg, **64**.
10. CHESTER, F. D. 1901. A Manual of Determinative Bacteriology, 352—353, MacMillan, New York.
11. GEDOELST, L. 1902. Les champignons parasites de l'homme et des animaux domestiques, **177**, Lierre, J. Van In et Cie., Bruxelles.
12. ROSENBACH, F. J. 1909. Z. Hyg. **63**, 343.
13. CASTELLANI, A. and CHALMERS, A. J. 1913. Manual of Tropical Medicine. 2nd Ed. 815, Baillière, Tindall and Cox, London.
14. WINSLOW, C.-E. A., BROADHURST, J., BUCHANAN, R. E., KRUMWIEDE, C., ROGERS, L. A., and SMITH, G. H. 1920. J. Bact., **5**, 191.
15. BUCHANAN, R. E. 1925. General Systematic Bacteriology, 163, Williams and Wilkins, Baltimore.
16. SCHÜTZ, J. W. 1886. Arb. Kaiserl. Gesundh. **1**, 56.
17. SMITH, T. 1895—'96. 12th and 13th Annual Reports of the Bureau of Animal Industry. U.S.D.A., 166—174.
18. LEHMANN, K. B., and NEUMANN, R. 1896. Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen bakteriologischen Diagnostik. 1st Aufl., **2**, 393, J. F. Lehmann, München.
19. CHESTER, F. D. 1897. Ann. Reports Delaware College Agr. Exp. Sta. **9**, 98.
20. PRETTNER, M. 1901. Berlin. tierärztl. Wochenschr. **669**.



21. TENBROECK, C. 1920. J. Exp. Med. **32**, 331.
22. TEICHMANN, J. 1922. Wien. tierärztl. Monatsschr. **9**, 433.
23. ZIBERT, S. 1922. Wien. tierärztl. Monatsschr. **9**, 396.
24. ZOSEL, O. 1922. Wien. tierärztl. Monatsschr. **9**, 408.
25. KLAUDER, J. V. 1926. J. Am. Med. Ass. **86**, 536.
26. HARKINS, M. J. 1927. J. Bact. **13**, 32.
27. KONDO, S. and SUGIMURA, K. 1935. J. Japan. Soc. Vet. Sci. **14**, 111.
28. KOHL, K. 1940. Vet. Arch. **10**, 500.
29. LANGFORD, G. C. 1952. Doctoral Dissertation, University of Maryland, College Park, Maryland.
30. BYRNE, J. L., CONNELL, R., FRANK, J. F., and MOYNIHAN, I. W. 1952. Canadian J. Comp. Med. **16**, 129.
31. BUCHANAN, R. E., ST. JOHN-BROOKS, R. and BREED, R. S. 1949. J. Gen. Microbiol. **3**, 444.
32. ZOFF, W. 1885. Die Spaltpilze, 88, 3rd Ed., Trewendt, Breslau.
33. KOCH, R. 1878. Untersuchungen über die Aetiologie der Wundinfektionskrankheiten, Vogel, Leipzig.

(Laboratory of Microbiology, Technological University, Delft, Holland).

# STUDIES ON TRUE DISSIMILATORY NITRATE REDUCTION

## I. FATE OF THE HYDROGEN DONATOR IN BACTERIAL NITRATE REDUCTION

by

**W. VERHOEVEN and J. J. C. GOOS**

(Received August 25, 1953).

### 1. INTRODUCTION

In an earlier publication (VERHOEVEN, 1952) the desirability of distinguishing various types of biological nitrate reduction has been stressed. There it has been proposed amongst others to use the term true dissimilatory nitrate reduction for those biological conversions of nitrate in which the nitrate is involved in an energy yielding process which, at least conditionally, is essential for the well-being and proliferation of the cell. For this true dissimilatory nitrate reduction it is already known since long that depending on the type of organism and on the external conditions the nitrate is converted into different reduction stages, such as nitrite, nitrous oxide, nitrogen or ammonia. Remarkably practically nothing is known of the fate of the organic substrates which function as hydrogen donator in the reduction process. It is usually accepted that these substrates are subject to a complete dehydrogenation to carbon dioxide. But, although the formation of the latter product has, indeed, often been established in a qualitative way, quantitative data regarding the fate of these hydrogen donators were until recently completely lacking. Yet it seemed worthwhile to investigate this point, since in the analogous processes of sulphate reduction (BAARS, 1930) and of carbonate reduction (BARKER, 1936) often incomplete dehydrogenation products of the organic substrates are formed. After the experimental part of the present investigation had been finished there appeared a publication by

SACKS and BARKER (1952) in which the same problem had been attacked. These authors made carbon balances of the denitrification caused by *Pseudomonas denitrificans* in a medium in which sodium succinate and glutamic acid were the only organic compounds present. The results were quite convincing: approximately 90% of the carbon of the organic substrates consumed was found back as carbon dioxide; the remaining 10% could be accounted for as the carbon present in the bacteria formed.

Since our investigations had been made both with a different bacterial species and a different substrate the publication of the results obtained seems justified with a view to the theoretical aspect of the problem.

## 2. METHODS.

The organism used in this study was strain 4 of *Pseudomonas aeruginosa* which had been found to be a typical denitrifier; under anaerobic conditions a profuse growth took place in nutrient broth to which 1%  $\text{KNO}_3$  had been added, the development being accompanied by a vigorous gas evolution.

From a young culture on broth agar, to which 1%  $\text{KNO}_3$  had been added, an inoculum was transferred into a sterile glass bottle completely filled with the medium and covered by a thin layer of liquid paraffine. The bottle was closed with a rubber stopper provided with a capillary glass tube through which the gas evolved was led into a gas burette. The medium consisted of tap water, 0.1%  $\text{K}_2\text{HPO}_4$ , 0.05%  $\text{MgSO}_4 \cdot 7 \text{ aq.}$  and varying concentrations of glucose and potassium nitrate. In all cases the pH was adjusted to a value between 7.0 and 7.2. The cultures were incubated at a temperature of 30° C. As soon as the gas development ceased — usually after 72 to 96 hours — the volume of the gas evolved was measured, after which both the gas and the medium were subjected to analysis. In all cases the gas proved to be a mixture of carbon dioxide, nitrogen and nitrous oxide. A sample of the medium was tested for the presence of nitrate and nitrite; in all successful experiments these reactions were found to be negative. Neither could any ammonia be detected. A second sample was used for the determination of carbon dioxide. Next the residual glucose was determined, and finally the amount of organic nitrogen formed was established with the aid of the Kjeldahl method.

We shall refrain from giving any data regarding the greater part

of the analytical methods employed, since they were the same as applied and carefully described by VERHOEVEN in his 1952 publication, to which the reader is referred.

As for methods not applied by VERHOEVEN we refer for the determination of carbon dioxide — free and bound — to the method described by BRAAK (1928), whilst for the determination of glucose the well-known iodometric method of SCHOORL and REGENBOGEN (1917) was applied.

### 3. NITROGEN BALANCES.

As until now most efforts to get an insight into the mechanism of nitrate reduction have mainly been restricted to qualitative observations, we decided to determine all nitrogen containing products quantitatively. Since, as already mentioned at the end of the experiment, the nitrate and the nitrite were invariably

TABLE I.

The fate of the nitrate nitrogen in true dissimilatory nitrate reduction by *Pseudomonas aeruginosa*.

Medium: tap water, 0.1%  $K_2HPO_4$ , 0.05%  $MgSO_4$  7 aq., 1% glucose and varying amounts of  $KNO_3$ , pH 7.2.

| Initial amount of $KNO_3$ present in mg eq. N | Nitrogen recovered in mg eq. N |       |        |                | Nitrogen recovered in % of nitrate consumed |       |        |
|-----------------------------------------------|--------------------------------|-------|--------|----------------|---------------------------------------------|-------|--------|
|                                               | Organic N                      | $N_2$ | $N_2O$ | Total recovery | Organic N                                   | $N_2$ | $N_2O$ |
| 5.2                                           | 0.4                            | 4.3   | 0.6    | 5.3            | 7.7                                         | 82.8  | 11.6   |
| 5.2                                           | 0.4                            | 4.3   | 0.5    | 5.2            | 7.7                                         | 82.8  | 9.6    |
| 5.2                                           | 0.4                            | 4.1   | 0.6    | 5.1            | 7.7                                         | 78.8  | 11.6   |
| 5.1                                           | 0.3                            | 4.2   | 0.3    | 4.7            | 5.9                                         | 82.4  | 5.9    |
| 5.1                                           | 0.3                            | 4.5   | 0.4    | 5.2            | 5.9                                         | 88.3  | 7.8    |
| 5.0                                           | 0.4                            | 4.1   | 0.4    | 4.9            | 8.0                                         | 82.0  | 8.0    |
| 5.0                                           | 0.3                            | 4.1   | 0.4    | 4.8            | 6.0                                         | 82.0  | 8.0    |
| 4.9                                           | 0.2                            | 4.1   | 0.4    | 4.7            | 4.1                                         | 83.8  | 8.2    |
| 5.0                                           | 0.3                            | 4.2   | 0.4    | 4.9            | 6.0                                         | 84.0  | 8.0    |
| 5.0                                           | 0.3                            | 4.2   | 0.4    | 4.9            | 6.0                                         | 84.0  | 8.0    |
| 5.0                                           | 0.3                            | 4.3   | 0.4    | 5.0            | 6.0                                         | 86.1  | 8.0    |
| 5.0                                           | 0.3                            | 4.4   | 0.4    | 5.1            | 6.0                                         | 88.0  | 8.0    |
| 4.7                                           | 0.5                            | 3.6   | 0.6    | 4.7            | 10.6                                        | 76.7  | 12.8   |
| 4.7                                           | 0.2                            | 4.1   | 0.4    | 4.7            | 4.3                                         | 82.3  | 8.5    |
| 9.6 <sup>1)</sup>                             | 0.7                            | 8.1   | 0.6    | 9.4            | 7.3                                         | 84.4  | 6.2    |

<sup>1)</sup> In this experiment the amount of glucose was raised to 2%.

completely consumed, the determinations could be restricted to a Kjeldahl nitrogen determination (organic N, mainly bacterial protein) of the medium and a volumetric determination and analysis of the gas evolved. The results of these experiments are collected in Table I.

From these results it is clear that *Pseudomonas aeruginosa* is able to convert large amounts of nitrate into the normal denitrification products  $N_2$  and  $N_2O$ . Next to these conversions a quite considerable part of the nitrate is apparently consumed for the building up of the bacterial protein. Though the percentage of  $N_2O$  in the denitrification gas is not as large as established by one of us (VERHOEVEN, 1952) in several experiments with *Denitrobacillus licheniformis*, it should be emphasized that it was always present in quantities of 10% or even more.

Also in contrast to the outcome of the last-mentioned experiments is the fact that nitrogen "losses" have not been observed. Apparently this result contrasts with those of KORSKOVA (1927) who working with *Pseudomonas aeruginosa* has reported such losses. However, it should be realized that the Russian investigator established these losses only in intermediate stages of the process and that at the end of her experiments all the nitrate nitrogen was practically found back in the gas evolved.

#### 4. CARBON BALANCES.

The next and main step in our experiments was the determination of the glucose consumed in the cultures and the attempt to recover the carbon initially present in the glucose. Qualitative experiments had left no doubt that carbon dioxide was at least one of the main products of the glucose conversion; it remained to be seen whether this would be the sole conversion product. From the beginning it was, however, clear that a quantitative recovery of the carbon of the glucose consumed in the carbon dioxide could not be expected, since evidently part of the glucose had been used up for the building up of the organic constituents of the bacterial cells originating in the cultures.

It seemed too cumbersome to collect in all experiments the bacteria finally present and to make a carbon determination in this material. We have, therefore, restricted ourselves to the following approximative procedure which seemed sufficiently accurate for our purpose. In a few experiments the nitrogen content of the dry



bacterial mass was determined and was in all cases found to be in the neighbourhood of 9.6%, indicating a protein content of the bacteria of about 60%. Accepting this to be the mean value, it seemed permissible to estimate in all experiments the dry weight of the bacteria from the organic nitrogen content of the medium. Moreover, on the basis of the data available, it seems safe to accept the carbon content of the bacteria to be 50% of the dry weight.

It is according to this procedure that the amount of carbon present in the bacteria, as indicated in Table II, has been assessed.

TABLE II.

The fate of the glucose carbon in true dissimilatory nitrate reduction by *Pseudomonas aeruginosa*.

Medium: tap water, 0.1%  $K_2HPO_4$ , 0.05%  $MgSO_4$  7 aq., 1% glucose and 1%  $KNO_3$ , pH 7.2.

| mg C in<br>glucose<br>consumed | mg C<br>recovered<br>in $CO_2$ | mg C<br>in<br>bacteria <sup>1)</sup> | mg C<br>recovered | Total<br>recovery<br>in % |
|--------------------------------|--------------------------------|--------------------------------------|-------------------|---------------------------|
| 106                            | 75.6                           | 29                                   | 105               | 99.2                      |
| 101                            | 71.6                           | 29                                   | 101               | 100                       |
| 106                            | 74.3                           | 29                                   | 103               | 97.6                      |
| 97                             | 69.0                           | 22                                   | 91                | 93.9                      |
| 100                            | 69.0                           | 22                                   | 91                | 91                        |
| 102                            | 70.2                           | 29                                   | 99                | 97                        |
| 101                            | 67.3                           | 22                                   | 89                | 88                        |
| 95                             | 66.4                           | 15                                   | 81                | 85.4                      |
| 92                             | 71.0                           | 22                                   | 93                | 101                       |
| 92                             | 70.3                           | 22                                   | 92                | 100                       |
| 92                             | 70.6                           | 22                                   | 93                | 101                       |
| 92                             | 71.8                           | 22                                   | 94                | 102                       |
| 107                            | 68.0                           | 36                                   | 104               | 97.4                      |
| 97                             | 68.0                           | 15                                   | 83                | 85.6                      |
| 195                            | 142                            | 50                                   | 192               | 99                        |

<sup>1)</sup> Assessed according to procedure given in text.

With due acknowledgement of the inaccuracy to which the figures in column 3 are subject the results obtained seem to guarantee the conclusion that the part of the glucose consumed which has not been used for the building up of the cell-material has been quantitatively converted into carbon dioxide and water. In other words the experiments leave no doubt that the denitrification process brought about by *Pseudomonas aeruginosa* with glucose

as hydrogen donator does not lead to a noticeable amount of incomplete dehydrogenation products. This result is in agreement with that obtained by SACKS and BARKER for a different bacterial species, using succinate and glutamate as a substrate. As has already been remarked in the Introduction the absence of incomplete dehydrogenation products in denitrification is, therefore, so remarkable, because this strongly contrasts with what has been shown to hold for the analogous processes of sulphate and carbonate reduction.

It seems, nevertheless dangerous to generalize the results under discussion, because we know of several instances in which of two related bacterial species one brings about a complete, and the second one an incomplete dehydrogenation of an organic substrate (acetic acid bacteria, sulphate reducing bacteria etc.). For this reason we have extended the experiments both to other bacterial species, and other substrates.

In this respect special attention was given to various alcohols. It should be remembered that BAARS in sulphate reduction, and BARKER in carbonate reduction established an almost quantitative conversion of ethanol into acetic acid. Using *Pseudomonas aeruginosa* partial success was obtained in so far as it proved possible to establish the presence of acetic acid, propionic acid and acetone as incomplete dehydrogenation products in media containing ethanol, propanol and iso-propanol respectively. With *Micrococcus denitrificans* also some acetic acid out of ethanol was formed. It should be added, however, that the amount of the said products corresponded only with a small fraction (2.5% maximal) of the substrates consumed. Nevertheless, these results bring a first indication that also in dissimilatory nitrate reduction the dehydrogenation occurs step-wise. Of course, there still remains the unsolved problem why in sulphate and carbonate reduction the incomplete dehydrogenations, and in nitrate reduction the complete hydrogenations prevail.

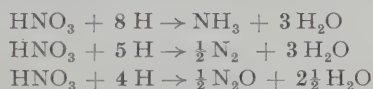
From Table II still one more conclusion may be derived, namely that on the average about 25% of the sugar consumed serves assimilatory purposes, whilst 75% is used up in the energy yielding process. It is interesting to compare these figures with those obtained in anaerobic sugar fermentations on the one hand, and in strictly aerobic metabolic processes on the other hand. In the case of sugar fermentation seldom more than 5-10% of the sugar is

used in assimilation, whilst the yield of cell material obtained under conditions of strict aerobiosis may rise to 50 or 60% of the sugar consumed. It is, therefore, evident that in this respect denitrification holds an intermediate position between fermentation and respiration, a result which might have been expected on the basis of an evaluation of the nature of the different processes.

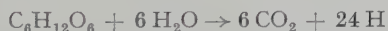
## 5. OXIDO-REDUCTION BALANCES.

The figures given in the two preceding chapters show that it has been possible to trace the final fate of the nitrate-nitrogen and of the glucose-carbon in an on the whole satisfactory way. It seemed indicated, however, also to check the reliability of the analyses made by drawing up an oxidoreduction balance. For it will be clear that the hydrogen consumed in the reduction of nitrate into the stages of ammonia — as occurring in the bacterial protein — nitrogen and nitrous oxide must have been derived from the dehydrogenation of glucose to carbon dioxide and water.

From the amounts of the various nitrate reduction products determined the equivalent amounts of hydrogen consumed in their formation can be calculated on the basis of the following equations:



The hydrogen consumed in these reactions must now have originated from the dehydrogenation of the glucose according to the equation:



The results of these calculations have been collected in Table III.

From Table III we may conclude that the hydrogen derived from the glucose converted into carbon dioxide in first approximation balances with the hydrogen consumed in the formation of the nitrate reduction products. Since, however, in all cases the latter figures surpass slightly the former ones, as clearly indicated by the fact that in the last column the figures surpass the theoretical value of 100%, it becomes probable that some systematic error in the analysis is involved. A possible explanation could be that the determination of the relatively small percentages of nitrous oxide in the denitrification gas by the combustion method after hydrogen addition has yielded too low results. This assumption does not

TABLE III.

Oxido-reduction balances in true dissimilatory nitrate reduction by  
*Pseudomonas aeruginosa*.

Medium: tap water, 0.1%  $K_2HPO_4$ , 0.05%  $MgSO_4$  7 aq., 1%  $KNO_3$ , 1% glucose, pH 7.2.

| mg eq. H<br>calculated<br>from glucose<br>converted<br>into $CO_2$ | mg eq. N     |       |        | mg eq. H consumed for the<br>reduction of nitrate to |       |        |                        | % H<br>ac-<br>counted<br>for |
|--------------------------------------------------------------------|--------------|-------|--------|------------------------------------------------------|-------|--------|------------------------|------------------------------|
|                                                                    | Organic<br>N | $N_2$ | $N_2O$ | Organic<br>N                                         | $N_2$ | $N_2O$ | Total<br>re-<br>covery |                              |
| 25.1                                                               | 0.4          | 4.3   | 0.6    | 3.2                                                  | 21.5  | 2.4    | 27.1                   | 108                          |
| 23.8                                                               | 0.4          | 4.3   | 0.5    | 3.2                                                  | 21.5  | 2.0    | 26.7                   | 112.1                        |
| 24.8                                                               | 0.4          | 4.1   | 0.6    | 3.2                                                  | 20.5  | 2.4    | 26.1                   | 105.1                        |
| 23.0                                                               | 0.3          | 4.2   | 0.3    | 2.4                                                  | 21.0  | 1.2    | 24.6                   | 106.9                        |
| 23.0                                                               | 0.3          | 4.5   | 0.4    | 2.4                                                  | 22.5  | 1.6    | 26.5                   | 115                          |
| 23.4                                                               | 0.4          | 4.1   | 0.4    | 3.2                                                  | 20.5  | 1.6    | 25.3                   | 108                          |
| 22.4                                                               | 0.3          | 4.1   | 0.4    | 2.4                                                  | 20.5  | 1.6    | 24.5                   | 109.2                        |
| 22.1                                                               | 0.2          | 4.1   | 0.4    | 1.6                                                  | 20.5  | 1.6    | 23.7                   | 107.2                        |
| 23.7                                                               | 0.3          | 4.2   | 0.4    | 2.4                                                  | 21.0  | 1.6    | 25.0                   | 105.3                        |
| 23.4                                                               | 0.3          | 4.2   | 0.4    | 2.4                                                  | 21.0  | 1.6    | 25.0                   | 106.8                        |
| 23.5                                                               | 0.3          | 4.3   | 0.4    | 2.4                                                  | 21.5  | 1.6    | 25.5                   | 108.3                        |
| 23.9                                                               | 0.3          | 4.4   | 0.4    | 2.4                                                  | 22.0  | 1.6    | 26.0                   | 108.8                        |
| 22.7                                                               | 0.5          | 3.6   | 0.6    | 4.0                                                  | 18.0  | 2.4    | 24.4                   | 107.4                        |
| 22.7                                                               | 0.2          | 4.1   | 0.4    | 1.6                                                  | 20.5  | 1.6    | 23.7                   | 104.2                        |
| 47.4                                                               | 0.7          | 8.1   | 0.6    | 5.6                                                  | 40.5  | 2.4    | 48.5                   | 102.1                        |

affect the nitrogen balances and the said errors might well account for the apparent "over-consumption" of hydrogen in the formation of the reduction products of the nitrate.

In the meantime we may not conclude this chapter without observing that in reality the hydrogen donated by the glucose should always surpass the hydrogen accounted for in the nitrate reduction products, since it is a well-known fact that the elementary composition of cell material is always slightly reduced as compared with that of a carbohydrate. This implies that in the conversion of glucose into bacteria in all cases some hydrogen is consumed. For this reason the % H accounted for in the balances should have been lower than 100.

Taking into account the rather satisfactory outcome of the nitrogen and carbon balances we cannot offer any explanation why this condition is not fulfilled in our analytical data.

### Summary.

The first aim of the investigations made has been the determination of the fate of glucose as a hydrogen donator in the dissimilatory nitrate reduction by *Pseudomonas aeruginosa*. The carbon balances made are strongly in favour of the view that the part of the glucose consumed which is not converted into cell material is completely converted into carbon dioxide and water. This conclusion is supported by the simultaneously made nitrogen and oxidoreduction balances.

The result should be considered as a confirmation of a widely held belief for which, however, until now experimental evidence was practically lacking.

### References.

- BAARS, J. K., 1930. Over sulfaatreductie door bacteriën. Thesis. Delft.  
BARKER, H. A., 1936. Arch. Mikrobiol. **7**, 404.  
BRAAK, H. R., 1928. Onderzoekingen over vergisting van glycerine. Thesis. Delft.  
KORSAKOVA, M. P., 1927. Bull. Acad. Sci. U.S.S.R. Sér. **6**, 1221.  
SACKS, L. E., and BARKER, H. A., 1952. J. Bact. **64**, 247.  
SCHOORL, N. and REGENBOGEN, A., 1917. Chem. Weekbl. **14**, 228.  
VERHOEVEN, W., 1952. Aerobic sporeforming nitrate reducing bacteria. Thesis. Delft.
-



(Stritch School of Medicine and Graduate School, Loyola University,  
Chicago 12, Ill.).

## THE FLAGELLATION AND TAXONOMY OF SPECIES OF *ACETOBACTER*

by

EINAR LEIFSON

(Received July 6, 1953).

The taxonomy of the genus *Acetobacter* and its various species has been based mainly on cultural and physiological characteristics. All motile species of the genus have been described by VAUGHN (1942) as having polar monotrichous flagella. In BERGEY's Manual of Determinative Bacteriology 6th Ed. (1948) the motile species are also described as having polar monotrichous flagella. My study of 30 strains of the genus, representing many of the commonly recognized species, failed to show any with polar monotrichous flagella. Two distinct types of flagellation were found: A polar multi-trichous type having from 3 to 8 flagella with unusually short wavelength, and a peritrichous type with rather few flagella of longer wavelength.

### EXPERIMENTAL DATA.

All of the cultures studied were received from outside sources and were regarded by the donors as typical species of the genus *Acetobacter*. Each culture was studied physiologically and culturally to confirm its identity according to the classification in BERGEY's Manual, 6th Ed. A culture was regarded as a species of *Acetobacter* if it had the following characteristics: rod shaped; gram negative; generally pleomorphic; motile or nonmotile; strictly aerobic; ability to oxidize glucose and ethyl alcohol to acids; scant growth in peptone media without added carbohydrate, alcohol, etc.; growth in media at pH 5 to 6 better than at pH 7.

According to VAUGHN and BERGEY's Manual, 6th Ed., the oxidation of acetic acid to CO<sub>2</sub> and H<sub>2</sub>O is of primary taxonomic importance for the separation of the species. Several basic media

were employed in this study using peptone and yeast extract in various concentrations and combinations. Liquid, semisolid and solid media were used. The acetate was added in concentration from 0.1% to 1.0%. Bromthymol blue and chlorphenol red were used as indicators. Overall best results were obtained in the following medium: Peptone 0.3%, yeast extract 0.2%, sodium acetate 0.2%, bromthymol blue 0.002%, pH 6.5. With some strains more rapid alkalinity was obtained with the medium in the form of agar slants. Incubation at 30°C. for two weeks was generally sufficient to bring out the final reaction. In the basic medium alone (i.e. without acetate), using Casitone as peptone, some strains of *Acetobacter* produce an acid reaction, some leave the medium neutral, and some produce a slightly alkaline reaction. Practically all species of gram negative bacteria which I have studied produce an alkaline reaction in this type of medium. If the acetate is oxidized to completion the medium becomes strongly alkaline. All of the strains tested, with one exception, gave comparable results with lactate substituted for acetate. The one exception was a strain labeled *A. mesoxydans vini* which produced an alkaline reaction in the lactate medium but not in the acetate medium.

The other differential tests employed in BERGEY's Manual need little comment. Ability to grow in a medium with ammonium salts as the sole source of nitrogen, such as Hoyer's solution, is used to differentiate *A. aceti* from *A. xylinum* and *A. rancens*. My experience did not confirm the usefulness of this test for the above purpose. All four of my strains of *A. xylinum*, having the characteristic leathery pellicle, grew well in Hoyer's solution. Serial transfer in the medium was not done and this might have given different results. Two of the cultures received as *A. suboxydans* produced a pinkish pellicle in glucose broth on prolonged incubation at 20°C. The pigment was limited to the bacterial growth and apparently water insoluble. I assume these to be strains of *A. roseum*. According to FRATEUR (1950) production of pink pigment is not a sound criterion for species differentiation. He suggests that it is better to label these strains as *roseum* varieties of some species. Following this suggestion my two cultures should probably be labeled *A. suboxydans* var. *roseum*. None of the cultures in my collection, including one labeled *A. oxydans* (ATCC 9433), grew as well or as rapidly at 21 as at 30°C., and a typical strain of this species is therefore not represented in the Collection. According to FRATEUR the original de-

scription of *A. oxydans* by HENNEBERG is insufficient for positive identification and he suggests that the organism is probably a variety of *A. suboxydans*.

Motility was determined by direct microscopic observation. The organisms were observed from a variety of media differing in composition, pH, temperature and length of incubation. The best motility was obtained in somewhat acid media, pH 5 to 6, and at incubation temperatures of 20 to 30°C. At 37°C. several strains were nonmotile which were motile at the lower temperatures. A typical strain of *A. aceti* (F-4) showed good motility at pH 6 but not at pH 7. In general the motility was better at 21° than at 30°C. Two types of motion were observed: A rapid, linear motion typical of polarly flagellated bacteria; and a wriggling motion typical of non-polar or peritrichously flagellated bacteria. The flagella were stained by the method of LEIFSON (1951) using glucose broth cultures. Good stains were more difficult to obtain than with most bacteria, possibly because of the high acidity of the medium, the presence of capsular material, and the tendency of all the strains to clump when washed in the centrifuge. 5% formalin was added to each culture and the organisms washed three times with distilled water using the centrifuge method. The flagellation and physiological reactions of the various strains are recorded in table I.

In figures 1 to 8 are reproduced photomicrographs showing the typical flagellation of several species. The polar multitrichous flagellation of *A. melanogenum* and *A. suboxydans* is unique in my experience. Based on the measurement of 20 flagella on separate organisms from each of 6 strains, a total of 120 measurements, the average wavelength was found to be 1.4  $\mu$ . Similar measurements of the flagella of several typical polar multitrichous *Pseudomonas* strains gave an average wavelength of 2.2  $\mu$ . Unless other bacteria are discovered in the future with similar shape and grouping of the flagella, a flagella stain alone is sufficient for identification of these species of *Acetobacter*. The peritrichous flagellation of *A. aceti*, *A. rancens* and *A. orleanense* is quite orthodox. The number of flagella per organism tends to be few and the arrangement is very uneven. The flagellation resembles that of the colon bacteria, *Agrobacterium* species, *Achromobacter* species, etc. Clumping on centrifugation is very pronounced and there are few individual organisms on the stained slides. The individual strains showed a greater variation in

TABLE I.

Some Comparative Characteristics of Species of the Genus *Acetobacter*.

| strain | received as                                     | reaction in<br>peptone acetate lactate |                   |                  | growth in<br>Hoyer's<br>medium | leathery<br>pellicle | pigment | catalase | flagellation | sug-<br>gested<br>genus |
|--------|-------------------------------------------------|----------------------------------------|-------------------|------------------|--------------------------------|----------------------|---------|----------|--------------|-------------------------|
| B-746  | <i>A. aceti</i>                                 | sl.alk.                                | v.alk.            | v.alk.           | —                              | —                    | —       | —        | peritrichous | <i>Acetobacter</i>      |
| B-1036 | <i>A. aceti</i>                                 | "                                      | "                 | "                | —                              | —                    | —       | —        | peritrichous |                         |
| F-4    | <i>A. aceti</i>                                 | "                                      | "                 | "                | +                              | —                    | —       | +        | peritrichous |                         |
| F-6    | <i>A. aceti</i> var. <i>muciparum</i>           | "                                      | "                 | "                | +                              | —                    | —       | +        | none         |                         |
| B-55   | <i>A. orleanense</i>                            | "                                      | "                 | "                | +                              | —                    | —       | +        | peritrichous |                         |
| B-679  | <i>A. acetum</i> var. <i>nairo-<br/>biense</i>  | "                                      | "                 | "                | ?                              | —                    | —       | +        | none         |                         |
| B-145  | <i>A. acetum-mocosum</i>                        | "                                      | "                 | "                | —                              | —                    | —       | +        | none         |                         |
| B-578  | <i>A. aceti</i>                                 | "                                      | "                 | "                | —                              | —                    | —       | +        | none         |                         |
| F-2    | <i>A. rancens</i>                               | "                                      | "                 | "                | —                              | —                    | —       | +        | none         |                         |
| F-5    | <i>A. rancens</i> var. <i>turbi-<br/>dans</i>   | "                                      | "                 | "                | —                              | —                    | —       | +        | peritrichous |                         |
| B-1025 | <i>A. turbidans</i>                             | "                                      | "                 | "                | —                              | —                    | —       | +        | none         |                         |
| H-277  | <i>A. rancens</i>                               | "                                      | "                 | "                | —                              | —                    | —       | +        | none         |                         |
| H-263  | <i>A. oxidans</i>                               | "                                      | "                 | "                | —                              | —                    | —       | +        | none         |                         |
| F-12   | <i>A. rancens</i> var. <i>saccharovorans</i>    | neutral<br>sl.alk.                     | sl.alk.<br>f.alk. | f.alk.<br>f.alk. | —<br>+                         | —<br>+               | —<br>—  | +        | none         |                         |
| F-3    | <i>A. xylinum</i>                               | "                                      | "                 | "                | +                              | +                    | —       | —        | none         |                         |
| F-9    | <i>A. xylinum</i> var. <i>maltoovorans</i>      | "                                      | "                 | "                | +                              | +                    | —       | —        | none         |                         |
| B-43   | <i>A. xylinum</i>                               | "                                      | "                 | "                | +                              | +                    | —       | +        | none         |                         |
| B-581  | <i>A. aceti</i>                                 | "                                      | "                 | "                | +                              | +                    | —       | +        | none         |                         |
| F-10   | <i>A. mesoxydans vini</i>                       | neutral                                | neutral           | sl.alk.          | —                              | —                    | —       | —        | none         |                         |
| F-14   | <i>A. mesoxydans</i> var. <i>saccharovorans</i> | "                                      | ?                 | neutral          | —                              | —                    | —       | —        | none         |                         |

TABLE I (continued).

| strain | received as                                              | reaction in<br>peptone acetate lactate |          | growth in<br>Hoyer's<br>medium | leathery<br>pellicle | pigment | catalase | flagellation        | sug-<br>gested<br>genus |
|--------|----------------------------------------------------------|----------------------------------------|----------|--------------------------------|----------------------|---------|----------|---------------------|-------------------------|
| F-1    | <i>A. suboxydans</i> var.<br><i>biourgianum</i>          | sl. acid                               | sl. acid | —                              | —                    | pink    | +        | polar multitrichous | <i>Acetomonas</i>       |
| F-16   | <i>A. suboxydans</i>                                     | "                                      | "        | —                              | —                    | pink    | —        | "                   |                         |
| F-15   | <i>A. suboxydans</i> var.<br><i>muciparum</i>            | "                                      | "        | —                              | —                    | —       | +        | "                   |                         |
| B-72   | <i>A. suboxydans</i>                                     | "                                      | "        | —                              | —                    | —       | +        | "                   |                         |
| B-1225 | <i>A. capsulatum</i>                                     | "                                      | "        | —                              | —                    | —       | +        | "                   |                         |
| B-1226 | <i>A. viscosum</i>                                       | "                                      | "        | —                              | —                    | —       | +        | "                   |                         |
| F-8    | <i>A. melanogenum</i> var.<br><i>maltovorans</i>         | "                                      | "        | —                              | —                    | brown   | —        | "                   |                         |
| F-13   | <i>A. melanigenum</i> var.<br><i>mallosaccharovorans</i> | "                                      | "        | —                              | —                    | brown   | +        | "                   |                         |
| B-58   | <i>A. melanogenum</i>                                    | "                                      | "        | —                              | —                    | brown   | +        | "                   |                         |
| B-63   | <i>A. melanogenum</i>                                    | "                                      | "        | —                              | —                    | brown   | +        | "                   |                         |
|        |                                                          | "                                      | "        | —                              | —                    | —       | —        | none                |                         |

Legend: sl. — slightly; f. — fairly; v. — very; alk. — alkaline; neutral — no change of reaction.



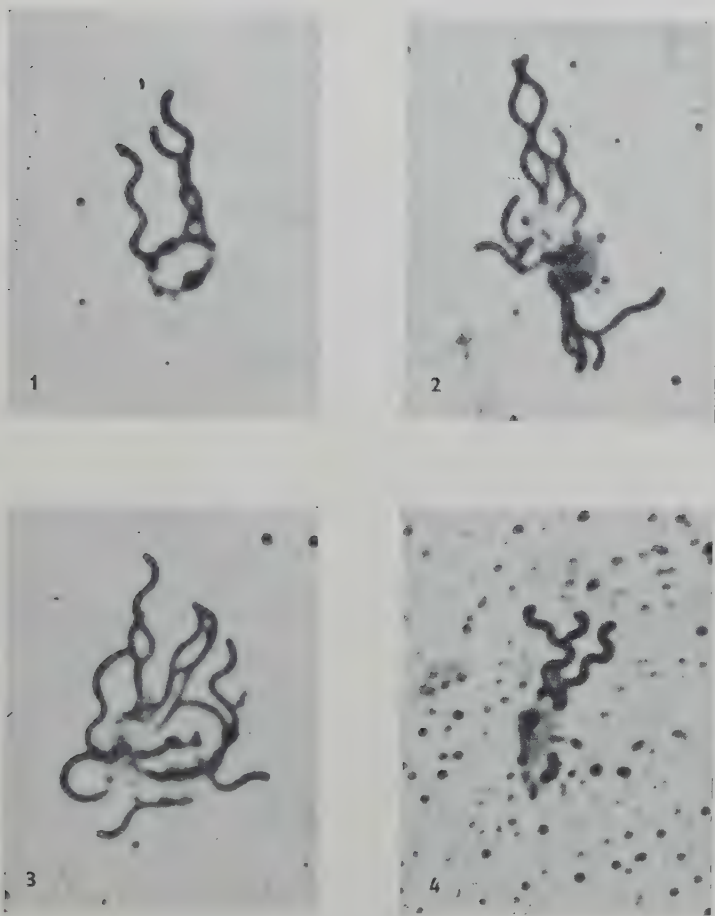


Fig. 1. *Acetobacter aceti* showing peritrichous flagella. Only the periphery of the soma took the flagella stain. The center was stained blue which shows light on the photograph. Leifson flagella stain. Photomicrograph  $\times 3600$ .

Fig. 2. *Acetobacter rancens* showing peritrichous flagella. Apparently a clump of two organisms. Leifson flagella stain. Photomicrograph  $\times 3600$ .

Fig. 3. *Acetobacter aceti* showing peritrichous flagella. Several organisms in one clump. Leifson flagella stain. Photomicrograph  $\times 3600$ .

Fig. 4. *Acetomonas suboxydans* var. *roseum* showing 3 polar flagella. Leifson flagella stain. Photomicrograph  $\times 3600$ .

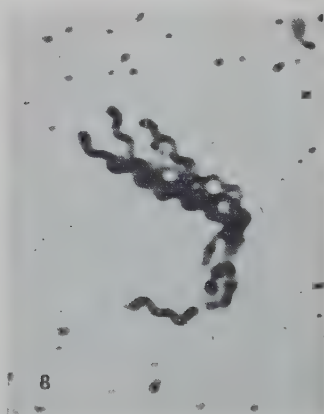
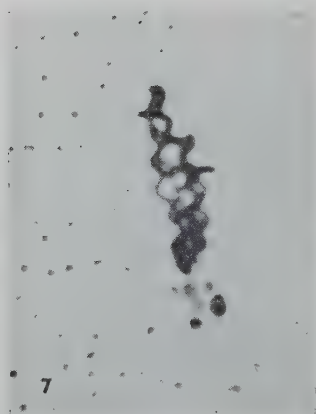
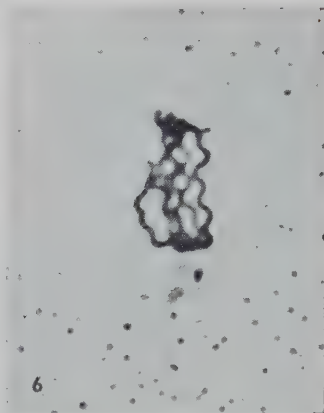
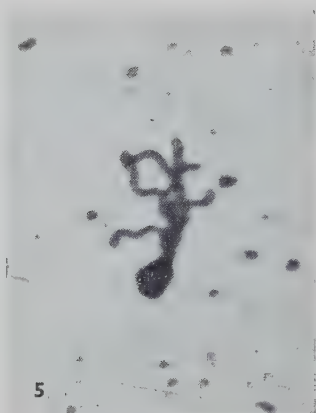


Fig. 5. *Acetomonas suboxydans* showing several polar flagella. The soma is atypical in staining solid with the flagella stain. Leifson flagella stain. Photomicrograph  $\times 3600$ .

Fig. 6. *Acetomonas melanogena* showing several polar flagella. The soma which was stained blue appears rather faintly as a vertical rod below the clump of flagella. Leifson flagella stain. Photomicrograph  $\times 3600$ .

Fig. 7. *Acetomonas melanogena* showing several polar flagella. The soma being stained blue appears rather faint on the photograph. Leifson flagella stain. Photomicrograph  $\times 3600$ .

Fig. 8. *Acetomonas suboxydans* showing the characteristic polar multitrichous flagellation of the one organism and one flagellum on the other. Leifson flagella stain. Photomicrograph  $\times 3600$ .



Fig. 9. *Pseudomonas* sp. showing polar multitrichous flagellation. Note the relatively much greater wavelength of these flagella compared to those of *Acetomonas*. Leifson flagella stain. Photomicrograph  $\times 3600$ .

wavelength of the flagella than was the case with the polar types. Based on the measurement of 20 flagella on separate organisms from each of 4 strains, the average wavelength was found to be  $2.9 \mu$ .

#### DISCUSSION.

The data presented in this paper confirm the soundness of previous classifications of the genus, such as that of VAUGHN. All of the motile strains studied which were able to oxidize acetate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  were found to be peritrichously flagellated. All of the motile strains studied which failed to oxidize acetate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  were found to have polar multitrichous flagella. In spite of certain cultural and physiological similarities it would seem taxonomically sound to separate the present genus *Acetobacter* into two genera. It is suggested that the generic name *Acetobacter* be retained for the species which oxidize acetate and/or lactate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , and which are peritrichously flagellated or nonflagellated. The logical type species would seem to be *A. aceti*. For the species which do not oxidize acetate or lactate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , and which have polar multitrichous flagella, or no flagella, the genus *Acetomonas* gen.nov. is suggested. *Acetomonas suboxydans* seems to be the logical type species. The genus *Acetomonas* seems most appropriately placed in the family *Pseudomonadaceae*, but the redefined *Acetobacter* genus seems more logically fitted into another family.

### S u m m a r y.

The *Acetobacter* genus as presently constituted include two distinctly different morphological types, one having peritrichous flagella with a wavelength averaging  $2.9\ \mu$  and the other having polar multitrichous flagella with a wavelength averaging  $1.4\ \mu$ . All of the peritrichously flagellated types actively oxidized acetic and lactic acids to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , while none of the polar flagellated types showed this property. It is proposed that the present *Acetobacter* genus be divided into two genera: *Acetobacter* and *Acetomonas* gen.nov. The redefined *Acetobacter* genus should include only peritrichously flagellated species and nonflagellated species with similar physiology. *Acetomonas* gen.nov. should include only polar multitrichous species and nonflagellated species of similar physiology. Typical species of the redefined *Acetobacter* genus should oxidize acetic or lactic acid to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  while typical species of *Acetomonas* gen.nov. should not have this property.

### A c k n o w l e d g m e n t s.

The author is greatly indebted to Prof. J. FRATEUR of the University of Louvain for providing 14 of the cultures studied; to WILLIAM C. HAYNES of the Northern Regional Research Laboratory, Peoria, Ill. for providing 14 of the cultures studied; and to Dr C. B. VAN NIEL of the University of California for providing one culture.

### R e f e r e n c e s.

- BREED, R. S., MURRAY, G. D. and HITCHENS, A. P. 1948. *Bergey's Manual of Determinative Bacteriology*, 6th Ed. Williams and Wilkins Co., Baltimore, Md. U.S.A.
- FRATEUR, J. 1950. *La Cellule* **53**, 287.
- LEIFSON, E. 1951. Staining Shape and Arrangement of Bacterial Flagella *J. Bact.* **62**, 377.
- VAUGHN, R. H. 1942. *Wallerstein Lab. Com.* **5**, 5.

(Laboratoire de Microbiologie, Université de Louvain, Héverlé-lez-Louvain, Belgique).

## ÉTUDE CHROMATOGRAPHIQUE DE CULTURES D'*ACETOBACTER* <sup>1)</sup>

par

**JOSEPH FRATEUR, PAUL SIMONART et TONY COULON**

(Reçu le 15 Avril 1953).

La récente classification des bactéries du genre *Acetobacter* (1) partage ces microorganismes en quatre groupes qui se distinguent les uns des autres par le pouvoir oxydant de leurs représentants. L'estimation de ce pouvoir oxydant tient compte notamment de l'oxydation de l'alcool éthylique en acide acétique, de l'oxydation de l'acide acétique en anhydride carbonique, de l'oxydation de la glycérine en dihydroxyacétone et de la formation d'acide gluconique ou d'acide 5-cétogluconique aux dépens de glucose. Ces diverses propriétés biochimiques concernent le ou les produits d'oxydation prépondérants dans le métabolisme de la bactérie lorsqu'elle est cultivée sur un substratum déterminé.

On relève, dans la littérature, la présence dans les cultures d'*Acetobacter* de nombreux métabolites. Bien qu'intéressantes, ces données sont trop fragmentaires et souvent insuffisamment prouvées pour pouvoir les utiliser dans la classification des bactéries du genre *Acetobacter* ou encore pour établir leur signification dans le chimisme de ces bactéries. Or il s'impose, autant pour compléter la systématique de ce genre que pour approfondir davantage nos connaissances sur le chimisme de ces bactéries, de faire une étude biochimique plus complète du métabolisme de toutes les espèces et variétés. Dans une telle étude, il s'agirait, en plus de la mise en évidence des métabolites telle qu'elle est appliquée avec succès pour la diagnose des espèces, de rechercher d'autres produits de métabolisme qui pourraient présenter du point de vue biochimique une importance

---

<sup>1)</sup> Travail fait sous les auspices de l'Union Belge des Vinaigriers, et de l'Institut pour la Recherche Scientifique dans l'Industrie et l'Agriculture.



particulière. Cet examen supposerait un temps et un travail considérable s'il devait être fait par les méthodes chimiques courantes et c'est pourquoi il s'indiquait d'étudier les cultures d'*Acetobacter* par chromatographie sur papier. Cette méthode d'investigation, à la fois expéditive et sensible, convient remarquablement bien pour une prospection biochimique telle que celle qu'il est souhaitable de faire dans le cadre de l'étude systématique des bactéries du genre *Acetobacter*. Après avoir mis au point une technique satisfaisante, les cultures des cent nonante huit souches d'*Acetobacter* dont nous disposons <sup>1)</sup>, ont été soumises à un examen par chromatographie sur papier dont les méthodes et les premiers résultats sont exposés dans la présente note, qui étudie surtout la production de substances cétoniques aux dépens de glucose et éventuellement aux dépens d'autres sucres ou de polyalcools. Ces métabolites à pouvoir réducteur, qui donnent une réaction de coloration avec l'aniline, présentent un intérêt particulier en égard aux propriétés cétogènes des *Acetobacter*. Quant à la production d'acides non réducteurs, elle fera l'objet d'une publication ultérieure.

## I. MÉTHODES EXPÉRIMENTALES.

### 1. Culture des *Acetobacter*.

On cultive les bactéries à 25° C., en erlenmeyer de 100 ml, contenant 25 ml d'eau de levure additionnée de 10% de sucre et de 3% de carbonate de calcium. Ces cultures se font en triple, et pour chaque souche, on examine la première culture après cinq jours, la seconde après dix jours et la troisième après quinze jours. Cependant, si le développement de la bactérie est trop lent, la dernière analyse se fera lorsque la culture est âgée de trente jours.

Au cours de ces périodes de culture, on veillera soigneusement à ne pas immerger le voile d'*Acetobacter*.

### 2. Analyse chromatographique.

On filtre la culture, pour la débarrasser de l'excès de carbonate de calcium; on acidifie le filtrat par addition d'acide oxalique jusqu'à pH = 2,0. On chromatographie sur papier à 15° C. par la méthode

<sup>1)</sup> Ces souches qui comprennent presque toutes les variétés décrites, proviennent soit de la collection du Laboratorium voor Microbiologie de Delft, soit d'isolements originaux de vinaigre, de bière, de fruits, etc. L'étude systématique de ces bactéries sera traitée dans une autre publication.

descendante, utilisant du papier filtre Whatmann N° 4, et du phénol saturé d'eau comme solvant; enfin, on révèle le chromatogramme à l'oxalate acide d'aniline (2). Dans ces conditions, les spots de glucose et d'acide 5-cétogluconique sont bruns, tandis que celui d'acide 2-cétogluconique est rouge. Le Rf respectif de ces substances à l'état pur dans le phénol/eau est voisin de 0,42, 0,15 et 0,20. Lorsque ces deux acides céto-gluconiques sont présents simultanément, leurs spots se chevauchent; l'identification reste cependant possible grâce à la couleur des spots plus qu'à leur Rf qui variera avec la composition de la solution examinée, surtout avec la teneur en glucose résiduel ainsi qu'avec la présence et la teneur en acide gluconique et en ses dérivés cétoniques. En plus des acides céto-gluconiques on peut encore éventuellement déceler d'autres substances réductrices réagissant avec l'aniline. En outre, dans les cultures d'*Acetobacter melanogenum* on décèle une substance qui, lors du séchage du chromatogramme (à 80°C.) donne un spot noir dont le Rf est voisin de 0,10. Quant à l'acide gluconique il ne réagit pas avec l'aniline, et ne peut être mis en évidence dans ces chromatogrammes. Enfin, pour chaque chromatogramme, on fait un essai à blanc pour le milieu de culture non ensemencé; celui-ci montre toujours en plus du sucre mis en oeuvre, un spot brunâtre de Rf plus élevé que celui du glucose; ce spot ne s'observe pas si l'on stérilise séparément le sucre et l'eau de levure.

## II. RÉSULTATS ET DISCUSSIONS.

L'examen des chromatogrammes obtenus dans les conditions qui viennent d'être décrites, et dont les figures 1—4 donnent quelques exemples tandis que le tableau I en résume les résultats, permet de faire pour chacun des quatre groupes d'*Acetobacter* — *peroxydants*, *oxydants*, *mesoxydants* et *suboxydants* — les observations suivantes.

### Groupe „peroxydants”.

Les chromatogrammes des trois souches d'*Acetobacter peroxydants* étudiées montrent que, dans les conditions culturelles appliquées, ces bactéries, aux dépens de glucose, ne forment pas, au moins en quantités décelables, de substances réductrices qui donnent des réactions de coloration avec l'oxalate acide d'aniline. Ceci est conforme à la description des deux espèces de ce groupe.

En outre, aucune substance réductrice, décelable chromatographiquement, n'est produite aux dépens de fructose, de mannose et de galactose, par la souche E.III.8.13.1.

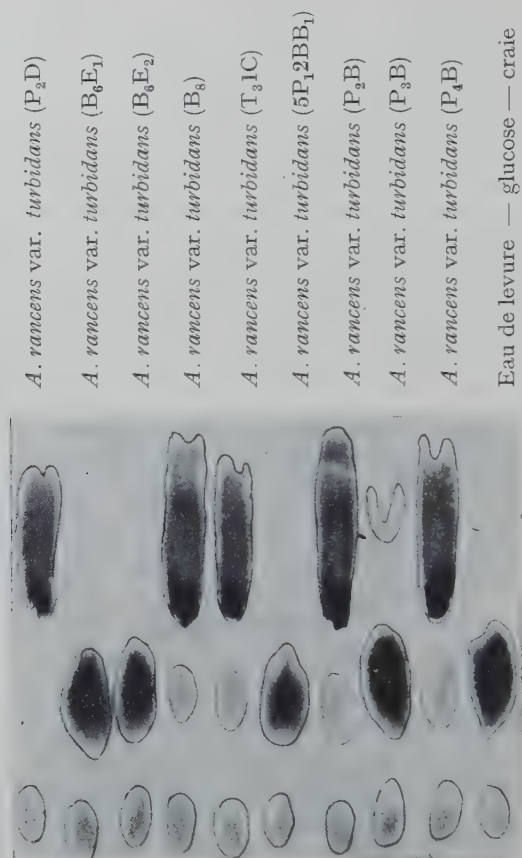


Fig. 1. Chromatogrammes de cultures de bactéries du groupe **oxydans**; on y voit le spot d'acide 2-cétogluconique formé aux dépens de glucose, par certaines variétés de ce groupe.

### Groupe „oxydans”.

L'étude chromatographique de ce groupe comprend l'examen de septante deux souches cultivées sur milieu glucosé, et de dix souches cultivées sur milieux fructosé, mannosé et galactosé.

Les chromatogrammes ainsi obtenus mettent en évidence la production d'acide 2-cétogluconique aux dépens de glucose, par plusieurs représentants de ce groupe. Cette propriété est inattendue d'après les caractéristiques biochimiques des bactéries du type

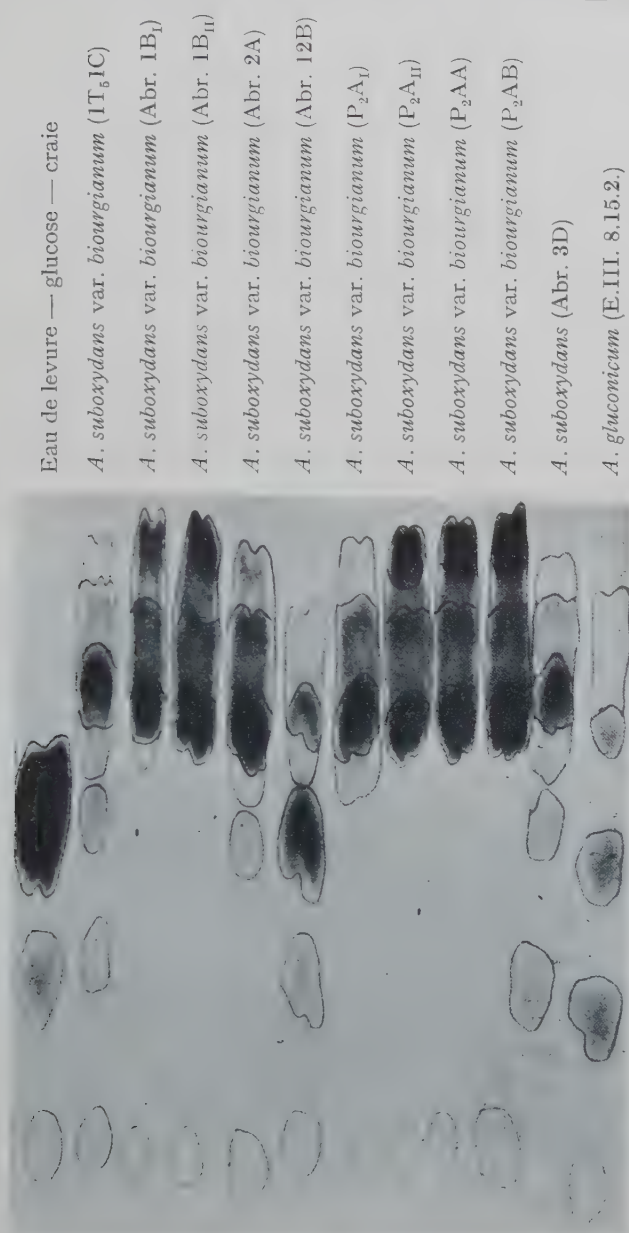


Fig. 2. Chromatogrammes de cultures de bactéries du groupe **suboxydans**; on y perçoit les spots d'acide 2-cétogluconique et d'acide 5-cétogluconique qui se chevauchent, et dont l'intensité de couleur indique la production abondante de ces acides aux dépens de glucose.

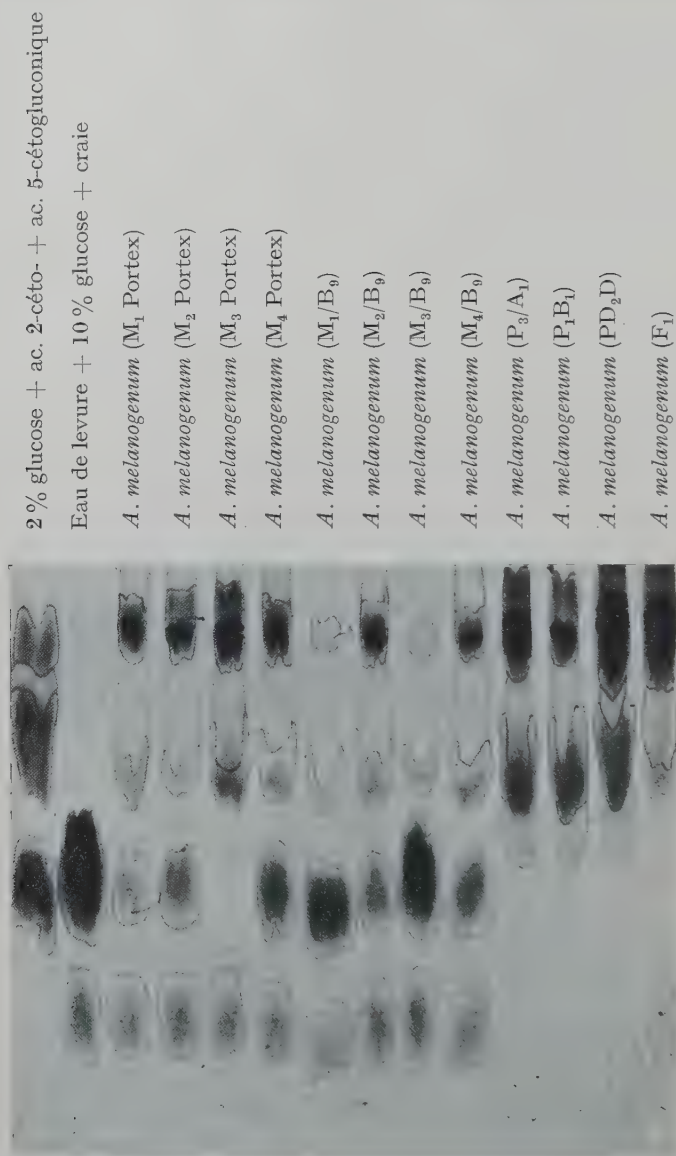


Fig. 3. Chromatogrammes de cultures d'*Acetobacter melanogenum* où l'on voit un spot noir caractéristique, de Rf plus petit que celui des spots d'acides cétogluconiques, produits aux dépens de glucose.



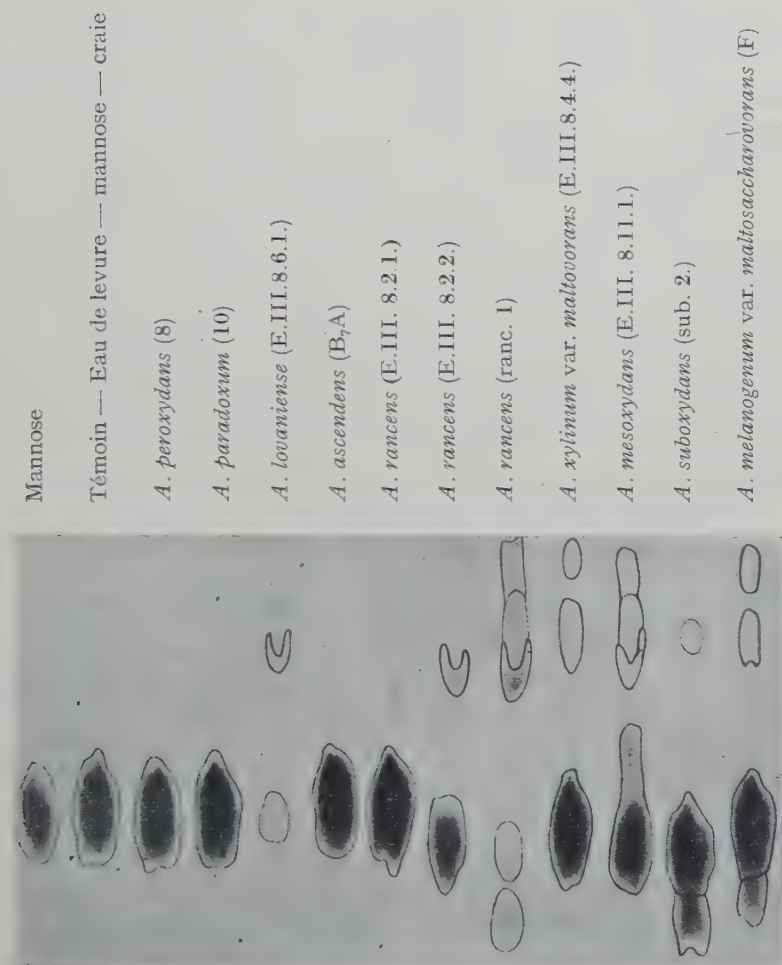


Fig. 4. Chromatogrammes de cultures de bactéries des divers groupes d'*Acetobacter* cultivées sur milieu mannosé, et où l'on perçoit la formation de substances réductrices par quelques variétés.

„oxydans”, qui ne forment pas d'acide 5-cétogluconique sur eau de levure-agar-glucose-craie et qui, dans leur ensemble, sont considérées comme acétogènes. Le pouvoir céto-gène n'est cependant pas totalement absent chez toutes les variétés de ce groupe car on admet (1) que certaines souches d'*Acetobacter rancens* sont capables dans des conditions culturelles déterminées de produire des

TABLEAU I.

| Noms —                                          | Désignation                                                                                                                                                                                                                                                                                                              | Chromatogrammes des cultures de 15<br>jours sur milieu glucosé <sup>1)</sup> |                     |            |                   |                 |
|-------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------|---------------------|------------|-------------------|-----------------|
|                                                 |                                                                                                                                                                                                                                                                                                                          | Spot noir                                                                    | Spot jaune brunâtre | Spot rouge | Spot jaune canari | Spot brun foncé |
| <b>Groupe peroxydants</b>                       |                                                                                                                                                                                                                                                                                                                          |                                                                              |                     |            |                   |                 |
| <i>A. peroxydants</i>                           | E.III.8.13.1, 5T <sub>1</sub> IC, 1T <sub>1</sub> IC                                                                                                                                                                                                                                                                     | —                                                                            | —                   | —          | —                 | ++++            |
| <b>Groupe oxydants</b>                          |                                                                                                                                                                                                                                                                                                                          |                                                                              |                     |            |                   |                 |
| <i>A. lovaniense</i>                            | E.III.8.6.1                                                                                                                                                                                                                                                                                                              | —                                                                            | —                   | —          | —                 | ++++            |
| <i>A. ascendens</i>                             | B <sub>1</sub> F <sub>1</sub> , B <sub>1</sub> F <sub>2</sub> , B <sub>1</sub> H <sub>1</sub> , B <sub>1</sub> P, B <sub>2</sub> I,<br>B <sub>3</sub> B <sub>2</sub> , B <sub>3</sub> C, B <sub>2</sub> 4, B <sub>6</sub> B, B <sub>7</sub> A,<br>B <sub>7</sub> B, B <sub>7</sub> A, B <sub>7</sub> C, B <sub>1</sub> 7 | —                                                                            | —                   | —          | —                 | ++++            |
| <i>A. rancens</i>                               | E.III.8.2.1, E.III.8.2.2,<br>B <sub>3</sub> J1, B <sub>3</sub> J11, 5P <sub>1</sub> 2BB3,<br>5P <sub>1</sub> 2BB2, P <sub>4</sub> A<br>Ranc 1, Ranc 2, B <sub>1</sub> D, B <sub>3</sub> G,<br>B <sub>3</sub> H, B <sub>4</sub> 3, B <sub>4</sub> 4, P9, Pêche<br>3A, Pêche 6, Pêche 3C                                   | —                                                                            | —                   | —          | —                 | —               |
|                                                 | B <sub>1</sub> Y                                                                                                                                                                                                                                                                                                         | —                                                                            | —                   | ++         | —                 | +               |
|                                                 | P <sub>1</sub> B                                                                                                                                                                                                                                                                                                         | —                                                                            | —                   | +          | —                 | ++              |
|                                                 | P <sub>1</sub> C                                                                                                                                                                                                                                                                                                         | —                                                                            | —                   | —          | —                 | ++              |
| <i>A. rancens</i> var.<br><i>turbidans</i>      | 5P <sub>1</sub> 2BB1, Abr. 1A<br>B <sub>6</sub> E <sub>1</sub><br>B <sub>6</sub> E <sub>2</sub><br>P <sub>3</sub> B<br>B <sub>2</sub> D, B8, T <sub>3</sub> IC, P <sub>2</sub> B, P <sub>4</sub> B,<br>P <sub>6</sub> C                                                                                                  | —                                                                            | —                   | —          | —                 | —               |
|                                                 |                                                                                                                                                                                                                                                                                                                          | —                                                                            | —                   | +          | —                 | —               |
|                                                 |                                                                                                                                                                                                                                                                                                                          | —                                                                            | —                   | ±          | —                 | —               |
|                                                 |                                                                                                                                                                                                                                                                                                                          | —                                                                            | —                   | ±          | —                 | +               |
|                                                 |                                                                                                                                                                                                                                                                                                                          | —                                                                            | —                   | ++         | —                 | —               |
| <i>A. rancens</i> var.<br><i>vini</i>           | 7, Voile 3AW <sub>3</sub> , Cal<br>Ca acet., Cay, Cas, Cax <sub>1</sub> ,<br>Cap <sub>1</sub> , Cap <sub>2</sub> , Caz, Vh                                                                                                                                                                                               | —                                                                            | —                   | ++         | —                 | —               |
|                                                 |                                                                                                                                                                                                                                                                                                                          | —                                                                            | —                   | ±          | —                 | —               |
| <i>A. rancens</i> var.<br><i>saccharovorans</i> | W <sub>2</sub> 2A, W <sub>2</sub> 2B <sub>1</sub> , W <sub>2</sub> 2C, W <sub>2</sub> 2B <sub>2</sub>                                                                                                                                                                                                                    | —                                                                            | —                   | +          | —                 | —               |
| <i>A. oxydants</i> var.<br><i>africanum</i>     | E.III.8.25.1                                                                                                                                                                                                                                                                                                             | —                                                                            | —                   | —          | —                 | ±               |
| <i>A. mucosum</i>                               | E.III.8.27.1                                                                                                                                                                                                                                                                                                             | —                                                                            | —                   | —          | —                 | ++++            |

|                                                          |                                                                                                                                                   |   |     |      |    |    |
|----------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|---|-----|------|----|----|
| <i>A. transcapsu-<br/>latum</i>                          | E.III.8.26.1                                                                                                                                      | — | —   | +++  | —  | ±  |
| <i>A. albuminosum</i>                                    | E.III.8.20.1                                                                                                                                      | — | —   | ++   | —  | ±  |
| <i>A. kuntzingia-<br/>num</i>                            | E.III.8.9.1                                                                                                                                       | — | —   | —    | —  | ++ |
| <b>Groupe</b>                                            |                                                                                                                                                   |   |     |      |    |    |
| <b>mesoxydans</b>                                        |                                                                                                                                                   |   |     |      |    |    |
| <i>A. aceti</i>                                          | E.III.8.1.1, E.III.8.1.2                                                                                                                          | — | +++ | +++  | —  | —  |
| <i>A. aceti</i> var.<br><i>muciparum</i>                 | E.III.8.1.3                                                                                                                                       | — | +++ | ++++ | —  | —  |
| <i>A. xylinum</i>                                        | E.III.8.4.1                                                                                                                                       | — | ±   | +++  | +  | —  |
|                                                          | DP <sub>2</sub>                                                                                                                                   | — | +   | ++   | +  | ±  |
|                                                          | B <sub>1</sub> C <sub>1</sub>                                                                                                                     | — | ++  | ++   | +  | —  |
|                                                          | W <sub>4</sub> A <sub>2</sub>                                                                                                                     | — | ++  | ++   | +  | +  |
|                                                          | W <sub>4</sub> B <sub>1</sub> , W <sub>4</sub> B <sub>2</sub> b                                                                                   | — | ++  | +++  | —  | —  |
|                                                          | W <sub>4</sub> B <sub>2</sub>                                                                                                                     | — | +++ | +++  | +  | —  |
|                                                          | Voile 3AW <sub>5</sub>                                                                                                                            | — | —   | +++  | +  | —  |
|                                                          | W <sub>6</sub> A                                                                                                                                  | — | +   | ++   | —  | ±  |
|                                                          | W <sub>6</sub> B                                                                                                                                  | — | +   | ±    | —  | —  |
|                                                          | Des 2, Des A, Des B                                                                                                                               | — | —   | ++   | —  | ±  |
|                                                          | MBA <sub>1</sub> , MBA <sub>2</sub> , MBA <sub>3</sub>                                                                                            | — | —   | ±    | —  | ±± |
|                                                          | MBB <sub>1</sub>                                                                                                                                  | — | —   | ±    | —  | +  |
| <i>A. xylinum</i> var.<br><i>xylinoides</i>              | Voile 3BW <sub>5</sub>                                                                                                                            | — | ±   | +++  | —  | —  |
|                                                          | 5P <sub>1</sub> 2BA                                                                                                                               | — | ++  | +++  | —  | —  |
|                                                          | E.III.8.4.3                                                                                                                                       | — | +   | +++  | —  | —  |
| <i>A. xylinum</i> var.<br><i>maltovorans</i>             | B3                                                                                                                                                | — | ±   | ++   | —  | —  |
|                                                          | Xyl B9                                                                                                                                            | — | ++  | +++  | —  | —  |
|                                                          | Xyl Portex                                                                                                                                        | — | +   | +++  | —  | —  |
| <i>A. mesoxydans</i>                                     | E.III.8.11.1                                                                                                                                      | — | ±   | ++   | —  | —  |
|                                                          | A4, A5, B3                                                                                                                                        | — | +   | +++  | —  | —  |
|                                                          | C, B <sub>6</sub> C                                                                                                                               | — | ++  | +++  | —  | —  |
|                                                          | 15                                                                                                                                                | — | +   | +    | —  | —  |
|                                                          | 17                                                                                                                                                | — | ±   | +    | —  | +  |
| <i>A. mesoxydans</i><br>var. <i>saccharo-<br/>vorans</i> | Voile 3CW <sub>2</sub>                                                                                                                            | — | —   | ±    | —  | +  |
|                                                          | E.III.8.11.2                                                                                                                                      | — | ±   | +    | —  | —  |
| <i>A. mesoxydans</i><br>var. <i>vini</i>                 | 3, Voile 2C <sub>II</sub> W <sub>2</sub>                                                                                                          | — | ±   | ++   | —  | ±  |
|                                                          | 4                                                                                                                                                 | — | ±   | ±    | —  | ±  |
|                                                          | 8, 20                                                                                                                                             | — | +   | +    | —  | ±± |
|                                                          | 18                                                                                                                                                | — | ±   | +    | —  | ±± |
|                                                          | 20 <sub>x</sub>                                                                                                                                   | — | ±   | ++   | ±  | ±  |
|                                                          | 20 <sub>x</sub> A20/7                                                                                                                             | — | —   | ++   | +  | ±  |
|                                                          | 20 <sub>x</sub> A22/8, Voile 2 <sub>II</sub> W <sub>2</sub> ,<br>Voile 2 <sub>III</sub> W <sub>2</sub> , Voile<br>2 <sub>III</sub> W <sub>2</sub> | } | —   | —    | ++ | —  |
|                                                          |                                                                                                                                                   |   | —   | —    | ++ | —  |

|                                                                          |                                                                                                                                                                                          |     |    |      |   |    |
|--------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|----|------|---|----|
|                                                                          | 20 <sub>x</sub> B20/7, Voile 1AIW <sub>2</sub> ,<br>Voile 3BW <sub>3</sub> , Voile 2W <sub>3</sub> ,<br>Voile 3A <sub>I</sub> W <sub>2</sub> , Voile 3A <sub>II</sub> W <sub>2</sub> }   | —   | —  | +    | — | ±  |
|                                                                          | W <sub>2</sub> A                                                                                                                                                                         | —   | +  | ++   | ± | ±  |
|                                                                          | W <sub>2</sub> B                                                                                                                                                                         | —   | +  | ++   | + | —  |
|                                                                          | W <sub>2</sub> C <sub>I</sub>                                                                                                                                                            | —   | +  | ++   | — | +  |
|                                                                          | Voile 2B <sub>II</sub> W <sub>2</sub> , Voile 2BW <sub>2</sub>                                                                                                                           | —   | —  | ++   | — | +  |
|                                                                          | Voile 2C <sub>I</sub> W <sub>2</sub>                                                                                                                                                     | —   | —  | ++   | — | ++ |
|                                                                          | W <sub>4</sub> A <sub>I</sub>                                                                                                                                                            | —   | —  | ±    | — | ±  |
| <i>A. orleanense</i>                                                     | E.III.8.14.1                                                                                                                                                                             | —   | —  | ++++ | — | —  |
| <i>A. dioxyacetoni-</i><br><i>cum</i>                                    | E.III.8.12.1                                                                                                                                                                             | —   | —  | ++   | — | —  |
| <i>A. dioxyacetoni-</i><br><i>cum</i> var. <i>pecti-</i><br><i>natum</i> | E.III.8.12.2                                                                                                                                                                             | —   | —  | —    | — | —  |
| <b>Groupe</b>                                                            |                                                                                                                                                                                          |     |    |      |   |    |
| <b>suboxydans</b>                                                        |                                                                                                                                                                                          |     |    |      |   |    |
| <i>A. suboxydans</i>                                                     | B5                                                                                                                                                                                       | —   | ±  | +++  | + | —  |
|                                                                          | Sub 1, Sub 2, Sub 3                                                                                                                                                                      | —   | —  | —    | — | —  |
|                                                                          | E.III.8.7.1                                                                                                                                                                              | —   | —  | +    | — | —  |
|                                                                          | B <sub>6</sub> F                                                                                                                                                                         | —   | ±  | ++++ | — | —  |
| <i>A. suboxydans</i>                                                     | B <sub>3</sub> B <sub>2</sub>                                                                                                                                                            | —   | ±  | ++++ | — | —  |
| var. <i>mucipa-</i><br><i>rum</i>                                        | Sub m.S.                                                                                                                                                                                 | —   | +  | ++   | — | —  |
|                                                                          | B <sub>1</sub> O                                                                                                                                                                         | —   | ++ | ±    | — | —  |
| <i>A. suboxydans</i>                                                     | Abr.1 2B, 1T <sub>5</sub> I.C                                                                                                                                                            | —   | —  | +++  | + | —  |
| var. <i>biourgia-</i><br><i>num</i>                                      | P <sub>2</sub> A <sub>II</sub> , P <sub>2</sub> AB, P <sub>2</sub> AA, P <sub>2</sub> A <sub>I</sub> ,<br>Abr.2A, Abr. <sub>I</sub> B <sub>I</sub> , Abr. <sub>I</sub> B <sub>II</sub> } | —   | —  | ++++ | — | —  |
|                                                                          | B <sub>2</sub> B                                                                                                                                                                         | —   | +  | ++++ | — | —  |
|                                                                          | Abr. 3D                                                                                                                                                                                  | —   | +  | +++  | — | —  |
| <i>A. gluconicum</i>                                                     | E.III.8.15.2                                                                                                                                                                             | —   | ±  | +    | + | —  |
| <i>A. melanogenum</i>                                                    | Mel 2P                                                                                                                                                                                   | +   | ++ | +++  | + | —  |
| var. <i>maltovo-</i><br><i>rans</i>                                      |                                                                                                                                                                                          |     |    |      |   |    |
| <i>A. melanogenum</i>                                                    | Mel 1P, M <sub>2</sub> B <sub>9</sub> , M <sub>4</sub> B <sub>9</sub>                                                                                                                    | +   | ++ | +++  | + | —  |
| var. <i>maltosac-</i><br><i>charovorans</i>                              | Mel 3P                                                                                                                                                                                   | +++ | ++ | +++  | + | —  |
|                                                                          | P <sub>3</sub> A <sub>I</sub> , P <sub>1</sub> BB, F, PD <sub>2</sub> D                                                                                                                  | +   | ±  | +++  | + | —  |
|                                                                          | M <sub>1</sub> B <sub>9</sub> , M <sub>3</sub> B <sub>9</sub>                                                                                                                            | ±   | ±  | ±    | — | —  |

- 1) Spot noir: substance non identifiée;  
 Spot jaune brunâtre: acide 5-cétogluconique;  
 Spot rouge: acide 2-cétogluconique;  
 Spot jaune canari: substance non identifiée;  
 Spot brun foncé: glucose.

traces de dihydroxyacétone et de fructose par oxydation de glycérol et de mannitol. Toutefois ce pouvoir cétogène très faible, ne se perçoit qu'irrégulièrement; il n'a pu être décelé, sur milieu glycérique, par les méthodes habituelles, dans les cultures en *en l'en m e y e r*, d'aucune des quarante-neuf souches examinées.

Dix variétés de ce groupe ont encore été cultivées sur milieux fructosé, mannosé et galactosé, et leurs cultures furent soumises à un examen chromatographique. Il en résulte que *A. albuminosum* (Delft, E.III.8.20.1) et *A. rancens* (Louvain, „Ranc" I) oxydent ces trois sucres avec formation de substances réductrices, tandis que *A. lovaniense* (Delft, E.III.8.6.1) produit aux dépens de fructose et de mannose des substances réductrices donnant une réaction colorée avec l'aniline; enfin, *A. transcapsulatum* (Delft, E.III.8.26.1) forme des substances réductrices par oxydation du galactose mais pas par oxydation des deux autres hexoses. Ces produits d'oxydation donnent entre autre un spot rouge dont le Rf est voisin de celui de l'acide 2-cétogluconique. A première vue il semble que ces corps réducteurs soient des dérivés cétoniques correspondants aux trois sucres étudiés, mais seules des déterminations chimiques plus complètes permettront de l'établir avec certitude.

D'autre part, dans les cultures d'*A. ascendens* (B<sub>7</sub>A), *A. rancens* (Delft, E.III.8.2.1 et E.III.8.2.2), *A. africanum* (Delft, E.III.8.25.1), *A. mucosum* (Delft, E.III.8.27.1) et *A. kuntzingianum* (Delft, E.III.8.9.1) on ne décèle, par les méthodes chromatographiques appliquées, aucune substance réductrice ni après dix, ni après quarante jours de culture sur milieux contenant soit du galactose, soit du mannose, soit du fructose.

Vu la production d'acide 2-cétogluconique par plusieurs représentants de ce groupe, il s'indiquait de rechercher de façon plus minutieuse si des céto-dérivés apparaissent dans les cultures de ces bactéries, lorsqu'elles se développent sur milieux glycéricinés ou mannités. Dans ce but, quarante-neuf souches du groupe oxydants furent cultivées en triple, en tubes à essais sur chacun des quatre milieux suivants: eau de levure + 2% mannitol, eau de levure + 2% mannitol + agar, eau de levure + 2% glycérol et eau de levure + 2% glycérol + agar. Pour chacune de ces séries, une première culture fut examinée, au point de vue de son pouvoir réducteur, par addition d'une solution de Fehling, après cinq jours, une seconde après dix jours et enfin la troisième après quinze jours d'incubation à 25° C.

D'autre part, pour chacune des quarante-neuf variétés, une cul-



ture en erlenmeyer de 100 ml contenant 20 ml d'eau de levure mannitée fut chromatographiée, après quinze jours d'incubation à 25°C.

Les résultats de ces divers essais sont réunis au tableau II qui reprend encore les indications obtenues par la chromatographie des cultures sur milieu glucosé, après quinze jours.

Il ressort soit de l'examen des chromatogrammes, soit des épreuves ordinaires pour rechercher le pouvoir cétogène que trente-sept souches ne produisent que de faibles traces soit d'acide 2-cétogluconique, soit de fructose, soit de dihydroxyacétone, soit encore de deux de ces substances. Par contre, douze souches, qui, par les critères habituels, devraient être rangées parmi les variétés d'*A. rancens* possèdent un pouvoir cétogène plus intense pour un ou deux des substrats qui viennent d'être cités. Les souches Ranc 1, Ranc 2, B 3H et B $\frac{1}{4}$  produisent de l'acide 2-cétogluconique aux dépens de glucose, mais on n'y décèle pas de substances réductrices aux dépens de mannitol; les trois premières forment de faibles traces de dihydroxyacétone tandis que la quatrième n'en produit pas du tout. Les souches B $\frac{4}{4}$ , „Pêche 3A", B $\frac{3}{4}$ , B $\frac{1}{4}$ D, P $\frac{9}{9}$ , „Pêche 3C", „Pêche 6" forment de l'acide 2-cétogluconique et du fructose; alors que les 2 premières produisent des traces de dihydroxyacétone, on ne retrouve pas ce céto-dérivé dans les cultures des cinq autres variétés. Enfin, les souches 5P $\frac{1}{1}$ 2BB $\frac{2}{2}$  et 5P $\frac{1}{1}$ 2BB $\frac{3}{3}$  oxydent le mannitol en fructose mais ne donnent pas de dérivés cétoniques aux dépens de glucose et de glycérol.

Il ressort de ces résultats, d'abord, que l'espèce *A. rancens* contient des variétés de pouvoir cétogène très varié ce qui pose le problème de la révision de cette espèce, du point de vue systématique. Il s'en dégage, ensuite, que le mannitol et le glucose conviennent mieux que le glycérol pour la mise en évidence du pouvoir cétogène.

### Groupe „mesoxydans”.

On sait que les bactéries de ce groupe possèdent un pouvoir cétogène bien développé; elles forment de la dihydroxyacétone, du fructose et de l'acide 5-cétogluconique aux dépens, respectivement, de glycérol, de mannitol et de glucose. Les chromatogrammes des cultures d'*Acetobacter* de ce groupe, obtenues sur milieu glucosé, montrent la présence d'acide 5-cétogluconique chez quarante-deux des soixante-six souches de ce groupe qui ont été étudiées. L'ab-

sence du spot d'acide 5-cétogluconique sur les chromatogrammes ne signifie pas que cet acide n'est pas formé par la bactérie étudiée; en effet, il y a lieu de tenir compte, d'abord, de la lenteur d'apparition de ce produit dans certaines cultures, et, ensuite, de son insolubilisation quasi totale sous forme de sel calcique, dans d'autres cultures. En outre, ces chromatogrammes indiquent que les soixante-six bactéries étudiées produisent toutes dans ces conditions de l'acide 2-cétogluconique.

Il s'ensuit d'abord que la production d'acide 2-cétogluconique par les *Acetobacter* est bien plus fréquente qu'on le supposait, même après la mise en évidence récente (3) de cet acide chez „*A. orleanense*”, „*A. pasteurianum*”, „*A. acetigenum*”, „*A. turbidans*” et „*A. acetosum*”, qui fait suite à la première identification (4) d'acide 2-cétogluconique dans des cultures d'„*A. gluconicum*” et d'„*A. suboxydans*”. Non seulement toutes les souches du groupe mesoxydants, et comme on le verra plus loin toutes celles du groupe suboxydants, produisent-elles de l'acide 2-cétogluconique, mais, en outre, quelques souches classées d'après les tests courants dans le groupe „oxydants” forment aussi cet acide. On est donc bien loin de l'opinion qui croyait pouvoir différencier le genre *Pseudomonas* du genre *Acetobacter* par l'absence d'acide 2-cétogluconique dans les cultures des ferments acétiques.

Il découle encore de ces résultats que la présence d'acide 2-cétogluconique est aussi générale que celle d'acide 5-cétogluconique dans les cultures des bactéries du groupe mesoxydants.

Il y a lieu aussi de souligner que les chromatogrammes de douze bactéries de ce groupe montrent encore un spot jaune canari, qui n'est pas identifié, et dont le Rf est voisin de 0,30.

Enfin, dans les cultures sur milieux fructosé, mannosé et galactosé on décèle chromatographiquement que l'une et l'autre souche oxydent facilement tel ou tel de ces sucres; c'est ainsi que *A. xylinum* var. *maltovorans* (Delft, E.III.8.4.4) oxyde le fructose et le galactose avec production de substances réductrices non identifiées, mais cette variété n'attaque guère le mannose; tandis que *A. acetii* (Delft, E.III.8.1.2) attaque plus facilement le mannose avec production de substance réductrice, qu'elle n'oxyde le fructose et le galactose; enfin *A. mesoxydans* (Delft, E.III.8.11.1) oxyde plus rapidement le galactose avec formation de substances réductrices, qu'elle n'oxyde le fructose et le mannose.

De même que pour les bactéries du groupe „oxydants”, les sub-

TABLEAU II.

Pouvoir cétoène des *Acetobacter* classés provisoirement dans le groupe *rancens*.

| Désignation                           | Chromatogrammes des cultures de 15 jours sur milieu glucosé <sup>1)</sup> |                     |            |                   |                 | Recherche de fructose dans les cultures sur milieu mannité |          |          |                            |          |          | Recherche, par son pouvoir réducteur, de dihydroxy-acétone dans les cultures sur milieu glycérriné |          |                            |          |          |   |
|---------------------------------------|---------------------------------------------------------------------------|---------------------|------------|-------------------|-----------------|------------------------------------------------------------|----------|----------|----------------------------|----------|----------|----------------------------------------------------------------------------------------------------|----------|----------------------------|----------|----------|---|
|                                       | Spot noir                                                                 | Spot jaune-brunâtre | Spot rouge | Spot jaune canari | Spot brun foncé | par pouvoir réducteur                                      |          |          |                            |          |          | cultures sur milieu liquide                                                                        |          | cultures sur milieu solide |          |          |   |
|                                       |                                                                           |                     |            |                   |                 | cultures sur milieu liquide                                |          |          | cultures sur milieu solide |          |          | 15 jours                                                                                           | 10 jours | 5 jours                    | 15 jours | 10 jours |   |
|                                       |                                                                           |                     |            |                   |                 | 5 jours                                                    | 10 jours | 15 jours | 5 jours                    | 10 jours | 15 jours |                                                                                                    |          |                            |          |          |   |
|                                       |                                                                           |                     |            |                   |                 |                                                            |          |          |                            |          |          |                                                                                                    |          |                            |          |          |   |
|                                       | Par chromatographie <sup>2)</sup>                                         |                     |            |                   |                 |                                                            |          |          |                            |          |          |                                                                                                    |          |                            |          |          |   |
| P <sub>4</sub> A                      | —                                                                         | —                   | —          | —                 | —               | —                                                          | —        | —        | —                          | —        | —        | —                                                                                                  | —        | —                          | —        | —        | — |
| Abr. 1A                               | —                                                                         | —                   | —          | —                 | —               | —                                                          | —        | —        | —                          | —        | —        | —                                                                                                  | —        | —                          | —        | —        | — |
| B <sub>3</sub> J1, B <sub>3</sub> J11 | —                                                                         | —                   | —          | —                 | —               | —                                                          | —        | —        | —                          | —        | —        | —                                                                                                  | —        | —                          | —        | —        | — |
| P <sub>3</sub> C                      | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| E.III.8.2.1                           | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| E.III.8.2.2                           | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| 5P <sub>1</sub> 2BB1                  | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| 5P <sub>1</sub> 2BB <sub>2</sub>      | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| 5P <sub>1</sub> 2BB <sub>3</sub>      | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| P <sub>3</sub> B                      | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| Cap 2                                 | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| B <sub>6</sub> E <sub>2</sub> , Cas   | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| Cap 1, Vh                             | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| Ca acet., Cay                         | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |
| Cax 1, Caz                            | —                                                                         | —                   | —          | —                 | —               | +                                                          | +        | +        | +                          | +        | +        | +                                                                                                  | +        | +                          | +        | +        | + |



stances réductrices formées par certains *Acetobacter* du groupe „mesoxydans”, aux dépens de fructose, de galactose et de mannose, donnent un spot sur les chromatogrammes, dont le Rf et la couleur rouge sont ceux de l'acide 2-cétogluconique; ceci suggère que l'on y a affaire aux 2-céto acides correspondants à ces sucres.

### Groupe „suboxydans”.

Sauf pour les variétés du type „*A. melanogenum*” les résultats de l'examen chromatographique des trente-deux souches du groupe *A. suboxydans* sont très voisins de ceux obtenus pour les bactéries du groupe précédent. On y rencontre partout de l'acide 2-cétogluconique mais en général en quantité plus abondante tandis que l'on ne décele chromatographiquement l'acide 5-cétogluconique que chez vingt-deux variétés. A ce sujet, la même remarque s'impose que celle faite (p. 122) en rapport avec l'étude du groupe „mesoxydans” et concernant l'absence du spot d'acide 5-cétogluconique.

Les diverses souches d'*A. melanogenum* étudiées présentent des chromatogrammes caractéristiques qui les différencient de toutes les autres bactéries étudiées. Sur ces chromatogrammes apparaît, lors du chauffage qui suit le développement chromatographique, un spot noir de Rf voisin de 0,1. La substance qui donne ce spot intervient probablement dans le phénomène de mélanogenèse qui est propre à cette espèce. En outre, un spot non identifié, coloré en jaune canari par l'oxalate acide d'aniline, dont le Rf = 0,30, et qui est identique à celui décelé chez les *Acetobacter* du groupe mesoxydans, se perçoit chez toutes les souches du type *A. melanogenum* sauf chez „M3B<sub>9</sub>”.

Cinq souches de ce groupe furent cultivées sur des milieux contenant soit du fructose, soit du mannose, soit du galactose, et leurs solutions de métabolisme furent chromatographiées. Il en résulte que le fructose et le mannose sont oxydés lentement par ces bactéries avec formation de produits réducteurs non identifiés tandis que le galactose paraît être oxydé plus facilement que les deux sucres précédents, avec production de substances réductrices. Il est possible que ces substances réductrices soient des acides 2-cétoniques; aussi serait-il intéressant d'étudier plus en détail, par la méthode chromatographique, le pouvoir, oxydant et en particulier le pouvoir céto-gène, non seulement des *Acetobacter* du groupe „suboxydans”, mais bien de tous les ferments acétiques en général, lorsqu'ils sont cultivés sur des sucres autres que le glucose.



Il est encore utile d'ajouter que sur les vingt-quatre souches cultivées sur fructose, aucune, d'après les observations chromatographiques, ne produit d'acide kojique. Mais, par contre, plusieurs variétés et notamment celles du type *melanogenum*, forment aux dépens du glucose une substance acide, qui, sur les chromatogrammes, donne un spot rouge avec le chlorure ferrique, analogue à celui de l'acide kojique, mais dont le  $R_f = 0,45$ , ce qui est sensiblement inférieur à celui de l'acide kojique, qui est voisin de l'unité.

Nous remercions très vivement ici le Professeur KLUYVER ainsi que le Dr VERHOEVEN et Mademoiselle WEISMAN du Laboratorium voor Microbiologie, Technische Hogeschool à Delft, non seulement pour l'envoi de nombreuses cultures d'*Acetobacter*, mais surtout pour les fructueuses discussions sur les problèmes exposés dans cette note. Celle-ci constitue un chapitre d'un plus vaste programme exécuté conjointement et qui couvre l'étude systématique du genre *Acetobacter*. Nous leur sommes très reconnaissants de cette précieuse collaboration.

### R é s u m é.

Cette note relate les résultats de l'examen, par chromatographie sur papier, de cent nonante huit souches de bactéries appartenant aux divers groupes du genre *Acetobacter*. Elle s'attache surtout à l'étude des substances réductrices, formées principalement aux dépens de glucose et accessoirement aux dépens de fructose, de mannose, de galactose, de mannitol et de glycerol.

Dans les cultures des bactéries du groupe „peroxydans", on ne décèle chromatographiquement aucun métabolite réducteur, ce qui est conforme à la description de ces bactéries. Par contre, chez plusieurs variétés, qui, d'après les critères habituels, doivent être classées dans le groupe „oxydans", on peut, chromatographiquement, mettre en évidence, de l'acide 2-cétogluconique.

Quant aux cultures des bactéries du groupe „mesoxydans" on y décèle chromatographiquement de l'acide 2-cétogluconique à côté d'acide 5-cétogluconique dont la formation aux dépens de glucose est générale chez les représentants de ce groupe. Ces deux acides se retrouvent aussi dans les cultures des bactéries du groupe „suboxydans". En outre, dans les cultures des bactéries du type „*melanogenum*" se décèle encore une substance donnant un spot noir de  $R_f$  voisin de 0,10, et qui intervient probablement dans le phénomène de mélanogenèse de ces bactéries, ainsi qu'une autre substance de  $R_f$  voisin de 0,45 et qui se colore en rouge par le chlorure ferrique.

Enfin, on peut mettre en évidence, d'une part, chez certaines variétés des groupes „mesoxydants" et „suboxydants", une substance donnant un spot de Rf voisin de 0,30 qui se colore en jaune canari par l'oxalate d'aniline, d'autre part, chez quelques souches de ces deux groupes ou encore du groupe oxydants, des substances réductrices, apparamment des acides cétoniques, formées aux dépens soit de fructose, soit de mannose, soit de galactose.

### Bibliographie.

1. FRATEUR, J. 1950. La Cellule **53**, 287.
  2. HORROKS, R. H. et MANNING, G. B. 1949. Lancet **256**, 1042.
  3. KULA, D., HALL, A. N. et WALKER, T. K. 1951. Nature **167**, 905.
  4. RIEDL-TUMOVA, E. et BERNHAUER, K. 1950. Biochem. Z. **320**, 472.
-

(Department of Bacteriology and Experimental Pathology, Netherlands' Institute for Preventive Medicine, Leiden, Holland).

## MIXED INFECTION WITH TYPE 2 POLIOMYELITIS VIRUS AND COLUMBIA SK GROUP VIRUS IN MAN

### AN ATTEMPT TO EXPLAIN ANY RELATION OF VIRUSES OF THE COLUMBIA SK GROUP TO HUMAN POLIOMYELITIS<sup>1)</sup>

by

**J. D. VERLINDE** and **J. H. MOLRON**<sup>2)</sup>

(Received September 30, 1953).

The relation, if any, of certain strains of the Columbia SK group to human poliomyelitis, is a puzzling matter. Both the original Columbia SK virus (9), and the MM strain (8) have been isolated from the central nervous system of rodents, which had been inoculated with either monkey cord containing the Yale SK strain of poliomyelitis virus, or medulla from a human case of bulbar poliomyelitis. Yet, the strains are different from the simian Yale SK strain, and from other rodent-adapted strains of poliomyelitis virus with regard to the antigenic composition, the host-range, the extremely high virulence for mice also following peripheral infection, and the haemagglutinating capacity. DICK (3) and MELNICK (10) suggested that the strains originated in rodents used for the adaptive process. JUNGBLUT (7), when disputing this view, argues that the concept of a latent infection in rodents is not in harmony with the high susceptibility of these animals for viruses of the Columbia SK group. He admits, however, the possibility that these viruses may have an active reservoir in certain species of resistant wild rodents. As a matter of fact, antibodies neutralizing the EMC virus, which also belongs to the Columbia SK group, have been demonstrated in wild rodents (18).

<sup>1)</sup> Aided with a grant from the National Health Research Council T.N.O., The Hague, and from the Dr Simon Baruch Foundation, New York.

<sup>2)</sup> W. H. O. Fellow, on leave of absence from the Université Libre de Bruxelles.

If the viruses of the Columbia SK group originate in animals, this does not exclude any pathogenicity for man. A laboratory infection of man with Mengo virus (4), and antibodies neutralizing viruses of the Columbia SK group have been demonstrated in human serum (5, 6, 10, 11, 17) and in pooled human gamma globulin (12). Moreover, human infections with viruses of the Columbia SK group have been shown by virological examination of human materials (1, 2, 17), and the development of antibodies neutralizing the patient's own virus have been demonstrated (16).

The present paper deals with a study on two virus strains isolated in this laboratory, which revives the problem of any relation of viruses of the Columbia SK group to human poliomyelitis.

#### THE A.K. AND E.S. STRAINS.

The A.K. strain was isolated in 1949 and the E.S. strain in 1952, both from the stools of children suffering from a paralytic syndrome clinically diagnosed as poliomyelitis.

The A.K. strain was carried through several monkey passages, rhesus as well as cynomolgus, before it was used for this study. In the 17th monkey passage, a peculiar observation was made. Cord from one rhesus monkey of the 16th passage produced paralysis in only 1 out of 3 rhesus monkeys, and it failed to produce paralysis in a subsequent rhesus passage. The 17th passage, however, proved virulent for mice on frequently repeated occasions, and under such carefully controlled conditions, and with adequate controls, that contamination or latent infection of the mice could be excluded (13, 15). The mouse-pathogenic strain proved avirulent for rhesus monkeys, and it had a very low virulence for cynomolgus monkeys.

In another 17th passage, starting from cord of a second monkey of the 16th passage, no evidence of pathogenicity for mice was obtained, and the strain was carried through 4 subsequent monkey passages without any difficulty.

The simian A.K. strain, in its 14th, 18th, and 19th passage, when typed in rhesus monkeys by the method of neutralization, could unequivocally be classified as a type 2 strain of poliomyelitis virus, whereas the murine A.K. strain possessed the pathogenic, haemagglutinating and immunologic characteristics of the Columbia SK group of viruses (14, 15).

The E.S. strain has been recovered repeatedly, and under care-

fully controlled conditions, which excluded either contamination or latent infection of the experimental animals, from stools and throat-washings from a patient. The patient's materials proved pathogenic for suckling-mice, older mice and cynomolgus monkeys. Cord from the first cynomolgus passage could be transmitted to cynomolgus and rhesus monkeys and to mice. Virus from mouse brain possessed the characteristics of the Columbia SK group of viruses (16). The simian strain, however, when being typed by the method of neutralization against the 3 prototype hyperimmune sera, appeared to be related to type 2 poliomyelitis virus (14).

In a preceding paper (15) a discrepancy between the results of typing of the simian A.K. strain by the method of neutralization in rhesus and in cynomolgus monkeys has been mentioned. This

TABLE 1.

Neutralization of the 19th passage of the simian A.K. strain and the second passage of the simian E.S. strain.

| Antiserum             | Dilution<br>of virus | Monkeys<br>paralyzed/inoculated |            | Virus |
|-----------------------|----------------------|---------------------------------|------------|-------|
|                       |                      | rhesus                          | cynomolgus |       |
| Lansing               | $10^{-1}$            | 2/2                             | 2/2        | A.K.  |
|                       | $10^{-2}$            | 0/4                             | 2/2        |       |
|                       | $10^{-3}$            | 0/2                             | 0/2        |       |
| Columbia SK           | $10^{-1}$            | 2/2                             | 2/2        |       |
|                       | $10^{-2}$            | 3/3                             | 2/2        |       |
|                       | $10^{-3}$            | 2/2                             | 1/2        |       |
| Lansing + Columbia SK | $10^{-1}$            |                                 | 1/2        |       |
|                       | $10^{-2}$            |                                 | 0/2        |       |
|                       | $10^{-3}$            |                                 | 0/2        |       |
| Normal monkey         | $10^{-1}$            | 2/2                             | 2/2        |       |
|                       | $10^{-2}$            | 2/2                             | 2/2        |       |
|                       | $10^{-3}$            | 2/2                             | 2/2        |       |
| Lansing               | $10^{-1}$            | 0/2                             | 3/4        | E.S.  |
|                       | $10^{-2}$            | 0/2                             | 2/2        |       |
| Columbia SK           | $10^{-1}$            | 2/2                             | 1/2        |       |
|                       | $10^{-2}$            | 1/2                             | 1/2        |       |
| Lansing + Columbia SK | $10^{-1}$            |                                 | 0/2        |       |
|                       | $10^{-2}$            |                                 | 0/2        |       |
| Normal monkey         | $10^{-1}$            |                                 | 2/2        |       |
|                       | $10^{-2}$            |                                 | 2/2        |       |



was suggested to be due to the presence of 2 components of the A.K. strain, one being of the Lansing type, the other of the Columbia SK type. A similar discrepancy was observed in typing experiments with the E.S. strain by the method of neutralization with prototype antisera. Table 1 shows the results of the neutralization reactions.

Lansing antiserum had an unequivocal neutralizing effect on both strains when tested in rhesus monkeys. No such effect was observed in cynomolgus monkeys, unless the Lansing immune serum had been mixed with an equal amount of Columbia SK immune serum. The discrepancy between the results of typing in rhesus and in cynomolgus monkeys can be explained, if one assumes, that both strains are mixed viruses, each possessing a Lansing and a Columbia SK component. Cynomolgus monkeys are susceptible for both type 2 poliomyelitis virus and Columbia SK group virus, so that either Lansing or Columbia SK serum fail to neutralize such a mixed virus. Rhesus monkeys, on the contrary, are usually highly resistant to strains of the Columbia SK group, so that neutralization with Lansing serum is effective.

If the strains under investigation were mixed viruses, a separation of the composing viruses must be possible by neutralization of limiting dilutions either with Lansing serum or with Columbia SK serum. Such a separation was easily performed in the case of the E.S. strain in its second cynomolgus passage.

The cords of the cynomolgus monkeys recorded in table 1, which had developed paralysis in the neutralization test with Lansing immune serum, produced a rapidly fatal encephalomyelitis in mice following intracerebral and intraperitoneal inoculation. The virus in the mouse brains possessed the characteristics of the Columbia

TABLE 2.

Neutralization of the E.S. strain from cord of monkeys, which had previously developed paralysis in a neutralization test with Columbia SK hyperimmune serum.

| Serum       | Dilution of virus | Number of cynomolgus monkeys paralyzed/inoculated | Comparison with the preceding passage (table 1) |
|-------------|-------------------|---------------------------------------------------|-------------------------------------------------|
| Lansing     | $10^{-1}$         | 0/2                                               | 3/4                                             |
|             | $10^{-2}$         | 0/2                                               | 2/2                                             |
| Columbia SK | $10^{-2}$         | 1/2                                               | 1/2                                             |

SK group. In contrast, the cords of the monkeys used in the neutralization test with Columbia SK immune serum, failed to produce paralysis in mice. A subsequent neutralization test in cynomolgus monkeys showed, that a suspension of these pooled cords was neutralized by Lansing immune serum (table 2).

Similar neutralization experiments were repeatedly carried out with the simian A.K. strain, but we failed constantly to produce paralysis in mice with cords from monkeys, which had developed paralysis following inoculation of mixtures of simian A.K. strain and either Lansing or Columbia SK immune serum.

Although our attempts to separate the presumed Columbia SK and type 2 poliomyelitis virus components of the simian A.K. strain have been unsuccessful, the discrepancy between the results of the neutralization test in rhesus and in cynomolgus monkeys indicates, that such components are present.

#### THE INTERFERENCE PHENOMENON.

The question, how the cord of a rhesus monkey of the 17th passage of the A.K. strain has become infected with a virus of the Columbia SK group remains unanswered. In contrast to the E.S. strain, in which case both type 2 poliomyelitis virus and Columbia SK group virus were isolated from the patient's material, which proved virulent for both monkeys and mice, a mouse-pathogenicity of the earlier passages of the A.K. strain has not been shown.

Rhesus monkeys proved highly resistant to the experimental infection with the murine A.K. strain. In particular, we failed to transmit 17th passage rhesus cord to other monkeys of the same species. An accidental contamination of this animal with the murine virus, which resulted in such a highly virulent cord for mice, is most unlikely. Another explanation is, that the original human material contained both viruses, which have been transmitted from monkey to monkey, in which the type 2 poliomyelitis virus has acted as an interfering agent, suppressing the development of the Columbia SK group virus, except in the 17th passage, when the latter was apparently the interfering agent, which suppressed the development of the type 2 poliomyelitis virus.

We have tried to reproduce this supposition experimentally by intracerebral passage of a mixture of the original Columbia SK virus, and the Aycock strain of type 2 poliomyelitis virus in rhesus monkeys. The diagram of these passages is presented in table 3,

which shows a striking similarity between the naturally occurring avirulence of the A.K. strain for rhesus monkeys, accompanied by the suddenly appearing mouse-pathogenicity, and the experimentally induced interference between the Aycock and the Columbia SK strains, accompanied by suppression of the Aycock strain. This experiment, however, does not provide any information as to the question whether the Columbia SK component has been carried through all passages, and whether or not it has already been present in the original human material.

TABLE 3.

Intracerebral serial passage of a mixture of the Aycock strain and Columbia SK virus, compared with passage of the 16th passage A.K. monkey cord.

| Aycock + Columbia SK              | A.K. 16th passage                 |
|-----------------------------------|-----------------------------------|
| ↓                                 | ↓                                 |
| 1 rhesus paralyzed                | 1 rhesus paralyzed, 2 negative    |
| ↓                                 | ↓                                 |
| 2 rhesus negative, mouse positive | 2 rhesus negative, mouse positive |
| ←      ↓                          | ←      ↓                          |
| rhesus negative, mouse positive   | rhesus negative, mouse positive   |

Another experiment shows also clearly this interference. The strains used are the 20th passage of the simian A.K. strain (type 2 poliomyelitis virus), and the 6th passage of the murine A.K. strain (Columbia SK group virus).

Rhesus monkeys were inoculated intracerebrally with 100 PD<sub>50</sub> of the simian A.K. strain, and either intracerebrally or intramuscularly with the murine A.K. strain (1 ml as a 10% suspension of mouse brain), either simultaneously, or after an interval of 3, 5, 7 and 9 days respectively. Moreover, rhesus monkeys were inoculated intramuscularly with 1 ml of a 10% suspension of the murine A.K. strain, followed by intracerebral inoculation of 100 PD<sub>50</sub> of the simian A.K. strain after an interval of 3, 5 and 7 days respectively. When the results were known, a similar interference experiment was performed with the murine A.K. strain and strains of each of the 3 types of poliomyelitis virus.

Table 4 shows, that intracerebral inoculation of the simian A.K. strain failed to produce paralysis and anterior horn lesions when the murine strain had been inoculated either simultaneously by intracerebral route, or simultaneously, and 3 and 5 days later by intramuscular route. No interference was observed when the inter-

TABLE 4.

Interference between the murine A.K. strain (AKM) and the simian A.K. strain (AKS) in rhesus monkeys.

| Rhesus | First (i.c.)<br>inoculation | Second (i.c. or i.m.)<br>inoculation | Interval<br>(days) | Paralysis |
|--------|-----------------------------|--------------------------------------|--------------------|-----------|
| 1035   | AKS                         | AKM i.c.                             | 0                  | +         |
| 1069   | "                           | " i.m.                               | 0                  | —         |
| 1070   | "                           | " i.m.                               | 0                  | —         |
| 1029   | "                           | " i.c.                               | 3                  | +         |
| 1071   | "                           | " i.m.                               | 3                  | —         |
| 1074   | "                           | " i.m.                               | 3                  | —         |
| 1075   | "                           | " i.c.                               | 5                  | +         |
| 1034   | "                           | " i.m.                               | 5                  | —         |
| 1080   | "                           | " i.m.                               | 5                  | —         |
| 1116   | "                           | " i.m.                               | 7                  | +         |
| 1117   | "                           | " i.m.                               | 7                  | +         |
| 1118   | "                           | " i.m.                               | 9                  | +         |
| 1119   | "                           | " i.m.                               | 9                  | +         |
| 1126   | Mahoney                     | AKM i.m.                             | 0                  | +         |
| 1127   | "                           | " i.m.                               | 0                  | +         |
| 1132   | "                           | "                                    | 0                  | +         |
| 1130   | Aycock                      | AKM i.m.                             | 0                  | —         |
| 1131   | "                           | " i.m.                               | 0                  | —         |
| 1134   | "                           | "                                    | 0                  | +         |
| 1128   | Leon                        | AKM i.m.                             | 0                  | +         |
| 1129   | "                           | " i.m.                               | 0                  | +         |
| 1133   | "                           | "                                    | 0                  | +         |
| Rhesus | First (i.m.)<br>inoculation | Second (i.c.)<br>inoculation         | Interval<br>(days) | Paralysis |
| 1135   | AKM                         | AKS                                  | 3                  | +         |
| 1136   | "                           | "                                    | 3                  | +         |
| 1137   | "                           | "                                    | 5                  | +         |
| 1138   | "                           | "                                    | 5                  | +         |
| 1139   | "                           | "                                    | 5                  | —         |
| 1140   | "                           | "                                    | 7                  | —         |

val between the first intracerebral and the second intramuscular inoculation was as long as 7 and 9 days.

In the reverse experiment, i.e. intramuscular inoculation of the murine strain and intracerebral inoculation of the simian strain,

TABLE 5.

Interference between the simian A.K. strain (AKS) and the murine A.K. strain (AKM) in mice.

Inoculated doses: AKM intracerebrally: 0.02 ml containing 100 LD<sub>50</sub>; intramuscularly: 0.1 ml containing 100 LD<sub>50</sub>; AKS: same amounts of a 10% suspension.

| First inoculation | Second inoculation | Interval (days) | Number of mice paralyzed/inoculated |
|-------------------|--------------------|-----------------|-------------------------------------|
| AKM i.c.          | AKS i.c.           | 0               | 4/4                                 |
| " i.c.            | " i.m.             | 0               | 4/4                                 |
| " i.c.            | " i.m.             | 1               | 4/4                                 |
| " i.c.            | " i.m.             | 2               | 4/4                                 |
| " i.p.            | " i.p.             | 0               | 4/4                                 |
| " i.m.            | " i.m.             | 0               | 4/4                                 |
| " i.m.            | " i.m.             | 1               | 4/4                                 |
| " i.m.            | " i.m.             | 2               | 4/4                                 |
| " i.m.            | " i.m.             | 3               | 4/4                                 |
| " i.m.            | " i.m.             | 4               | 4/4                                 |
| " i.m.            | " i.m.             | 5               | 4/4                                 |
| " i.p.            | " i.m.             | 0               | 0/4                                 |
| " i.p.            | " i.m.             | 1               | 4/4                                 |
| " i.p.            | " i.m.             | 2               | 3/4                                 |
| " i.p.            | " i.m.             | 3               | 2/4                                 |
| " i.p.            | " i.m.             | 4               | 4/4                                 |
| " i.p.            | " i.m.             | 5               | 4/4                                 |
| AKS i.m.          | AKM i.c.           | 0               | 4/4                                 |
| " i.m.            | " i.c.             | 1               | 4/4                                 |
| " i.m.            | " i.c.             | 2               | 3/4                                 |
| " i.m.            | " i.c.             | 3               | 4/4                                 |
| " i.m.            | " i.c.             | 4               | 4/4                                 |
| " i.m.            | " i.c.             | 5               | 4/4                                 |
| " i.m.            | " i.c.             | 7               | 4/4                                 |
| " i.m.            | " i.p.             | 0               | 4/4                                 |
| " i.m.            | " i.p.             | 1               | 0/4                                 |
| " i.m.            | " i.p.             | 2               | 4/4                                 |
| " i.m.            | " i.p.             | 3               | 4/4                                 |
| " i.m.            | " i.p.             | 4               | 4/4                                 |
| " i.m.            | " i.p.             | 5               | 4/4                                 |
| " i.m.            | " i.p.             | 7               | 4/4                                 |
| " i.m.            | " i.m.             | 0               | 4/4                                 |
| " i.m.            | " i.m.             | 1               | 4/4                                 |
| " i.m.            | " i.m.             | 2               | 4/4                                 |
| " i.m.            | " i.m.             | 3               | 4/4                                 |
| " i.m.            | " i.m.             | 4               | 4/4                                 |
| " i.m.            | " i.m.             | 5               | 4/4                                 |
| " i.m.            | " i.m.             | 7               | 4/4                                 |
| " i.p.            | " i.p.             | 0               | 4/4                                 |
| " i.p.            | " i.p.             | 2               | 4/4                                 |
| " i.p.            | " i.p.             | 3               | 4/4                                 |
| " i.p.            | " i.p.             | 4               | 4/4                                 |
| " i.p.            | " i.p.             | 5               | 4/4                                 |
| " i.p.            | " i.p.             | 7               | 4/4                                 |



with intervals of 3 and 5 days, no interference occurred. When, however, the interval was 7 days the monkeys were protected, presumably as a result of immunity against the simian strain induced by the murine strain, which is in accordance with the results of earlier immunity experiments (13, 15).

The murine A.K. strain had no interfering effect upon infection with the Mahoney and Leon strains. Interference, however, was observed between the murine A.K. strain and the Aycock strain. A similar phenomenon was demonstrated between the original Columbia SK strain and the Aycock strain by JUNGEBLUT and SANDERS (9).

Whereas the murine A.K. strain had a marked interfering effect on the intracerebral infection with the simian strain in rhesus monkeys, such an effect of the simian strain on infection of mice with the murine strain was, although present, much less striking, apparently because of the very high virulence of the murine strain for mice (table 5). Only then, when either AKM was inoculated intraperitoneally, and AKS intramuscularly, or AKS was inoculated intramuscularly, and AKM intraperitoneally, some interference occurred when the interval was 0 and 1 day respectively.

#### DISCUSSION.

In a limited number of cases of human poliomyelitis, the causative agent, a type 2 poliomyelitis virus, has been claimed to be associated somehow with a strain of the Columbia SK group of viruses. The problems of the discovery of the original Columbia SK virus by transmission of the simian Y-SK strain to rodents, and of the isolation of the MM strain from rodents, which had been inoculated with central nervous system from a fatal case of poliomyelitis have never been settled conclusively. The experiments with the more recently isolated Dutch strains A.K. and E.S. may throw more light on this problem.

The study of these strains indicates, that at least in the case of E.S., a mixed infection of the patient with a type 2 poliomyelitis virus, and a strain of the Columbia SK group has occurred. There is no conclusive evidence that the same has been true in the case of A.K., although it does not seem unlikely in the light of the experience with the E.S. strain. The repeated isolation of the latter proves, that the animal species, which are infected with the original

material, are important factors in the determination of the nature of the virus. If only rhesus monkeys had been used in the isolation experiments, or if typing had been carried out in this animal species, the Columbia SK group virus would presumably have disappeared, and only a type 2 strain of poliomyelitis virus would have been identified. If, on the other hand, only mice had been used, nothing but a virus of the Columbia SK group would have been isolated. The evidence obtained from isolation experiments in mice and in monkeys, that the E.S. strain could be a mixture of at least 2 viruses, was confirmed by the neutralization tests in cynomolgus monkeys, but not in rhesus monkeys.

The A.K. strain had been carried through many monkey passages. In the 17th passage a virus of the Columbia SK group could be shown, on many times repeated occasions, in the cord of a rhesus monkey. This observation was a peculiar one, for two reasons. First, none of the earlier monkey passages, as far as they have been examined, proved pathogenic for mice; second, the presence of a virus of the Columbia SK group in a rhesus monkey is a very unusual finding, in particular because of the fact, that this strain proved avirulent when transmitted to other rhesus monkeys. Neutralization experiments with different passages of the A.K. strain revealed a type 2 strain of poliomyelitis virus when typed in rhesus monkeys, whereas typing in cynomolgus monkeys presented evidence of a mixed virus. We failed, however, to separate the components by neutralization of limiting dilutions with either Lansing or Columbia SK hyperimmune serum.

Since a marked interference between the simian and the murine A.K. strain could be shown, the suggestion could be made, that the inability to separate the two strains might be attributable to this phenomenon. Perhaps, the Columbia SK component, which appeared suddenly in the 17th monkey passage, has suppressed the multiplication of the simian component, which is a type 2 poliomyelitis virus. This view is supported by experimental evidence.

Another, theoretical, explanation for the unsuccessful attempts to separate the two components, might be found in the assumption that the A.K. strain is a hybrid of type 2 poliomyelitis virus and Columbia SK virus, which has, in monkeys, the pathogenic characteristics of type 2 poliomyelitis virus, and the immunologic characteristics of both components.

The explanation of the phenomenon by interference, leading to suppression of one component through successive monkey passages, seems to us the most acceptable one. It is striking, that the Columbia SK group strain interfered only with type 2 poliomyelitis virus, and not with the other types, under experimental conditions.

### S u m m a r y.

Two virus strains isolated from stools of poliomyelitis patients were apparently a mixture of a type 2 strain of poliomyelitis virus, and of a strain of the Columbia SK group of viruses. Both components of the E.S. strain could be separated by neutralization of second passage cynomolgus monkey cord with either Lansing or Columbia SK hyperimmune serum. A similar technique with the 19th monkey passage of the A.K. strain failed to separate the components of this strain, which is suggested to be due to interference. Under experimental conditions, the murine A.K. strain was shown to interfere with the simian A.K. strain. Similarly, the original Columbia SK virus interfered with the Aycock strain of type 2 poliomyelitis virus. No interference was observed between the murine A.K. strain and types 1 and 3 poliomyelitis virus.

### R e f e r e n c e s.

1. K. BELLER und W. KELLER, *Klin. Woch.* **27**, 422, 1949.
2. R. BIELING und F. KOCH, *Z. Kinderheilk.* **72**, 85, 1952.
3. G. W. A. DICK, *J. Immun.* **62**, 375, 1949.
4. G. W. A. DICK, A. M. BEST, A. J. HADDOW and K. C. SMITHBURN, *Lancet* II, 286, 1948.
5. B. HOFMAN und C. W. JUNGEBLUT, *Klin. Woch.* **30**, 1013, 1952.
6. C. W. JUNGEBLUT, *Ann. Pediatr.* **67**, 519, 1950.
7. C. W. JUNGEBLUT, A. M. A. *Archives Path.* **52**, 18, 1951.
8. C. W. JUNGEBLUT and G. DALLDORF, *Amer. J. Publ. Health* **33**, 169, 1943.
9. C. W. JUNGEBLUT and M. SANDERS, *J. exp. Med.* **72**, 407, 1940.
10. J. L. MELNICK, *Bact. Reviews* **14**, 233, 1950.
11. J. E. SMADEL and J. WARREN, *J. Clin. Invest.* **26**, 1197, 1947.
12. J. D. VERLINDE, *Ned. Tijdschr. v. Geneesk.* **97**, 929, 1953.
13. J. D. VERLINDE, B. BEEM and A. KLARENBECK, *Antonie van Leeuwenhoek* **18**, 364, 1952.
14. J. D. VERLINDE, B. HOFMAN and E. NIHOUL, *Bull. World Health Org.* (in print).
15. J. D. VERLINDE and A. KLARENBECK, *Antonie van Leeuwenhoek* **19**, 324, 1953.

16. J. D. VERLINDE and H. A. E. VAN TONGEREN, Arch. Virusforsch. (in print).
  17. O. VIVELL und R. MAUER, Z. Imm.forsch. **109**, 246, 1952.
  18. J. WARREN, S. B. RUSS and H. JEFFRIES, Proc. Soc. exp. Biol. a. Med. **71**, 376, 1949.
-

[Centre de Collections de Types Microbiens de Lausanne (Suisse) et Laboratoire de Pathologie des Insectes, Institut National de la Recherche Agronomique, Alès (France)].

## RÉSISTANCE À LA LYOPHILISATION, DU VIRUS DE LA POLYÉDRIE DE *THAUMETOPOEA PITYOCAMPA* SCHIFF (LEPIDOPTERA)

par

**C. VAGO** et **P. HAUDUROY**

(Reçu le 29 juin 1953).

La conservation à l'état vivant et virulent des virus des granules, polyédries et d'autres viroses d'Insectes est un problème capital aussi bien pour les recherches sur ces viroses que pour les collections de microorganismes.

En effet, il est indispensable que la matière virulente employée pour les recherches soit toujours comparable et que l'on puisse effectuer de longues séries d'essais avec une même souche étudiée et déterminée. Pour les collections, l'impossibilité de culture de ces virus sur milieux artificiels ne permet pas de réaliser un maintien par repiquages successifs.

Etant donné ces faits, il était indiqué d'envisager pour la conservation des ultravirus des polyédries, l'application du principe de la „lyophilisation” qui a déjà permis la conservation de la virulence et de la faculté de développement d'autres groupes de microorganismes et notamment de plusieurs ultravirus (1).

Avant d'évaluer la durée de conservation de la virulence par la lyophilisation, évaluation qui consiste en des contrôles renouvelés pendant 4—5 ans au plus, il était essentiel de déterminer si les ultravirus résistaient à l'opération de la lyophilisation elle-même.

Les essais ont porté sur l'ultravirus de la polyédrie du Lépidoptère *Thaumetopoea pityocampa* Schiff mise en évidence et étudiée récemment (2).



La matière virulente a été obtenue à partir d'un élevage de larves de ce Lépidoptère dans lequel une importante mortalité par polyédrie a été provoquée par synergie d'une infection artificielle „per os” et des conditions favorables à la pathogénèse réalisées à l'aide d'une chambre climatisée spéciale.

Les individus morts étant reconnus par examen microscopique, porteurs de corps d'inclusion, furent broyés et mélangés à la solution physiologique de Ringer jusqu'à obtention d'une suspension homogène. Celle-ci a été débarrassée des souillures par filtration à travers une toile fine permettant cependant le passage des corps d'inclusion polyédriques dont certains ont un diamètre atteignant jusqu'à 6 microns.

La suspension a été stabilisée avec contrôle numéral des corps d'inclusion sous concentration de 10,000 000 polyèdres visibles à  $900 \times$  par  $\text{mm}^3$ . La moitié de cette solution a été soumise à la lyophilisation.

Ce procédé est théoriquement simple. Sa mise au point pratique l'est infiniment moins et les difficultés rencontrées par les constructeurs sont évidemment à l'origine des multiples appareillages proposés et, bien souvent, des échecs totaux ou partiels observés.

Après avoir analysé, pendant plusieurs années, au Centre de Collections de Types Microbiens de Lausanne les différents temps de l'opération, nous avons mis au point un prototype qui nous donne toute satisfaction et nous a permis d'obtenir en particulier les résultats rapportés ici.

L'obtention du vide se fait à l'aide de deux étages de pompes. La première est une pompe à palettes qui amorce l'étage secondaire, constitué par une pompe à vapeur de mercure.

Sur la tête de cette pompe sont montés, en étoile, des bras, munis chacun de tétines auxquelles on reliera les ampoules contenant le matériel à lyophiliser. La liaison entre tétine et col de l'ampoule est faite par des caoutchoucs imperméables aux gaz. Chaque bras est muni d'un robinet qui permet de le relier au système de vide ou de l'isoler. Entre la pompe à palette et la pompe à diffusion se trouve placé le condenseur constitué par un récipient de quelques litres plongé dans un Dewar spécial contenant de la neige carbonique et de l'acétone et maintenu, par conséquent, pendant toutes les opérations à  $70^\circ\text{C}$ . environ au dessous de zéro. L'ensemble de l'appareil comprend un indicateur de vide, des appareils de contrôle et de sécurité divers.

La chaleur de vaporisation est prise dans l'air de la pièce et il suffit de maintenir celle-ci à 20—25°C. pour obtenir des résultats excellents et rapides.

Les ampoules dont nous nous servions sont des ampoules spéciales de quelques centimètres cubes à fond très large et plat et à col central ou desaxé: ce qui permet d'obtenir des surfaces d'évaporation très grandes.

Les opérations furent conduites de la façon suivante. Le virus à lyophiliser est d'abord mélangé à un protecteur (substance de nature albuminoïde variable avec le matériel soumis à l'opération), placé stérilement dans les ampoules stériles qui sont alors montées sur les rampes séparées du vide. Les pompes sont en marche depuis quelque temps déjà. Le contenu des ampoules est congelé brusquement par immersion dans un mélange de neige carbonique et d'acétone. La rampe est mise en liaison avec le vide. Après quelques instants l'air „libre” contenu dans les ampoules ou les rampes est éliminé. La sublimation commence et, suivant la quantité de matériel à lyophiliser, sera terminée en une ou plusieurs heures. Jamais le temps de lyophilisation ne dépasse quatre ou cinq heures (plusieurs centaines d'ampoules contenant chacune 2 cc). Lorsque l'opération est terminée (ce qu'il est facile de juger par la stabilisation du vide à  $10^{-3}$ ,  $10^{-4}$  et par le fait que les ampoules ont une température égale à celle de la pièce), les ampoules sont scellées sous un vide aussi poussé que possible, ce qui est à notre avis une condition absolument essentielle pour la conservation de la vitalité des bactéries ou des virus.

Le contrôle de l'effet de lyophilisation a consisté en l'infection de larves saines 144 heures après la lyophilisation. Celle-ci a été effectuée par administration „per os” de la matière lyophilisée remise en suspension dans les proportions initiales.

Les chenilles tests provenaient de Génolhac (GARD), elles étaient au 4e âge et furent maintenues avant et après l'infection dans des conditions atmosphériques reconnues favorables à la pathogénèse de cette polyédrie, conditions réalisées en chambre climatisée.

Le contrôle a été basé sur deux séries de témoins, l'une sans aucune infection, l'autre infectée avec la suspension fraîche de virus dont la moitié a servi pour la lyophilisation.

Quinze jours après l'infection nous constatons les taux de mortalité par polyédrie suivants:

TABLE 1.

| Traitement                                        | Infection avec<br>suspension<br>lyophilisée |    |    |      | Témoin I<br>Infection avec<br>suspension non<br>lyophilisée |    |    |    | Témoin II<br>sans infection |    |    |    |
|---------------------------------------------------|---------------------------------------------|----|----|------|-------------------------------------------------------------|----|----|----|-----------------------------|----|----|----|
|                                                   | 1                                           | 2  | 3  | 4    | 1                                                           | 2  | 3  | 4  | 1                           | 2  | 3  | 4  |
| Lots N°                                           |                                             |    |    |      |                                                             |    |    |    |                             |    |    |    |
| Nombres de<br>larves                              | 25                                          | 50 | 25 | 30   | 25                                                          | 25 | 50 | 40 | 40                          | 25 | 40 | 50 |
| Nombre de lar-<br>ves mortes par<br>polyédrie     | 13                                          | 27 | 16 | 19   | 16                                                          | 14 | 39 | 28 | 0                           | 0  | 0  | 0  |
| Pourcentage des<br>larves mortes<br>par polyédrie | 52                                          | 54 | 64 | 63,3 | 64                                                          | 56 | 78 | 70 | 0                           | 0  | 0  | 0  |

Ces observations montrent d'abord, en comparant la série „lyophilisée” avec la série Témoin II, que la matière lyophilisée peut déclencher l'apparition de la polyédrie. Le taux d'apparition est élevé, la durée de l'incubation est inférieure à 15 jours.

Ensuite, la comparaison avec la série Témoin I détermine un pourcentage de mortalité de même grandeur entre infection avec matière lyophilisée et non lyophilisée. Les écarts de variation de la mortalité dans les lots sont également du même ordre.

Ces constatations permettent de considérer que les virus contenus dans une suspension de corps d'inclusion de la polyédrie de *Thaumetopoea pityocampa* Schiff résistent à l'opération de la lyophilisation. Cette précision favorable en elle-même pour l'emploi de la lyophilisation pour le maintien des souches d'ultravirus d'insectes est en même temps le point de départ des examens de contrôle de la conservation de la virulence au cours de divers délais après la lyophilisation.

### References.

1. HAUDUROY, P. 1952. Liste des êtres microscopiques conservés dans les collections de cultures types. Centre de Collections de Types microbiens, Lausanne.
2. VAGO, C. 1953. La polyédrie de *Thaumetopoea pityocampa* (nouvelle maladie à ultravirus d'insectes). Ann. des Epiph. En cours d'impression.

(From the Laboratory of Hygiene, University of Amsterdam).

## INHIBITION OF GROWTH OF TUBERCLE BACILLI BY ALI-ESTERASE INHIBITORS IN VARIOUS CULTURE MEDIA

by

**A. CHARLOTTE RUYS**

(Received July 11, 1953).

### INTRODUCTION.

The present communication is concerned with the effects of inhibition of the ali-esterase in tubercle bacilli. Ali-esterases are enzymes which hydrolyse preferentially simple aliphatic esters such as triacetin, tributyrin and methyl butyrate. They are present not only in animal tissues but also in lower forms of life, e.g. seeds, bacteria and neoplastic cells. However, the natural substrates and the physiological functions of these enzymes are not known (8).

MENDEL and co-workers (5—9) showed that in rats an almost complete inhibition of the ali-esterase (tributyrinase) activity of serum, liver, intestinal mucosa and testis does not produce any noticeable ill-effects. This discovery raised the question whether in the course of evolution the ali-esterases might have lost much of their importance for the animal kingdom, while retaining their essential role in the metabolism of the lower forms of life. Should this suggestion of MENDEL's prove true, then inhibition of ali-esterase activity might be a means of interfering selectively with the metabolism and growth of microorganisms and other parasitic cells. Evidence substantiating this hypothesis was obtained by MENDEL, MYERS, UYLDERT, RUYS and DE BRUYN (7) in experiments on germinating seeds, a lymphosarcoma and mycobacteria *in vitro*. It was shown that alkyl phosphate derivatives such as diethyl p-nitrophenyl phosphate (E 600, paraoxon) and diisopropyl fluorophosphonate (DFP) inhibit the ali-esterase activity of tubercle bacilli and that the concentrations that inhibit their ali-esterase also inhibit their growth in the medium of Dubos. Further experiments

have now been carried out to study (a) the influence of the nutrient medium in which the tubercle bacilli are growing, and (b) the reversibility of the growth inhibition by E 600.

#### MATERIALS AND METHODS.

A series of experiments with various culture media was carried out with the strain H37Rv of *M. tuberculosis* typus humanus. As noted previously (7), similar results are obtained with tubercle bacilli freshly isolated from human sputa. The nutrient medium of Dubos contained albumin from human plasma in place of bovine albumin (2). The results of previous experiments with this medium (7) were compared with the results obtained after omitting Tween 80 and with the results obtained after adding 2% glycerol to the medium. Similar experiments were carried out with the nutrient medium of Proskauer and Beck, which contains 2% glycerol but no albumin or Tween 80.

In order to test the effects of the ali-esterase inhibitor E 600 in a more physiological medium, a series of experiments was performed with sputum smears on glass slides in the medium of Price (10). This medium consists of human blood collected in glucose-citrate solution and lysed with an equal volume of water. Cultures of tubercle bacilli were not suitable for these experiments, because it proved impossible to obtain smears in which the bacilli lay isolated from each other on the slides. Therefore a positive sputum was spread evenly over a number of small glass slides; these were dried in the air, treated with 4%  $\text{H}_2\text{SO}_4$  for 6 minutes, washed in distilled water and placed in small flasks containing various concentrations of E 600 in haemolysed blood. For each concentration of E 600 a set of 6 to 8 flasks containing one slide per flask was used. At intervals of 2 or 3 days one or two slides from each concentration of E 600 were washed and stained by Ziehl-Neelsen's or Hallberg's nightblue method.

#### RESULTS.

In the experiments mentioned above, it had been shown that in the ordinary medium of Dubos E 600 inhibits the growth of tubercle bacilli in concentrations from 0.05 mg% upwards (7). When Tween 80 was omitted from the medium a much stronger concentration of E 600 was needed to obtain the same effect. Concentrations of 0.3 and 1 mg% E 600 proved to be practically ineffective, although



the same concentrations had been found to inhibit completely the growth of tubercle bacilli in the presence of Tween 80 for several weeks. However, concentrations above 5 mg% did inhibit the growth in the absence of Tween (Table I) and a concentration of 14 mg% suppressed nearly all growth during at least 6 weeks. This concentration of E 600 did not kill the bacteria; when they were subcultured on the 31st day in media without inhibitor, they grew at a normal rate. Moreover, the inhibition of growth was not due to breakdown products of E 600; high concentrations of p-nitrophenol, e. g. 3 mg%, are not harmful to tubercle bacilli in the medium with or without Tween.

TABLE I.

Effect of E 600 in the medium of Dubos without Tween on cultures of tubercle bacilli grown in ordinary Dubos medium.

| Days after inoculation | E 600 (mg%) |        |      |       |
|------------------------|-------------|--------|------|-------|
|                        | 0           | 1      | 5    | 10    |
| 3                      | +           | +      | +    | +     |
| 4                      | ++          | ++     | ++   | +     |
| 5                      | +++         | ++(+)  | ++   | +     |
| 8                      | +++(+)      | +++(+) | +++  | ++    |
| 10                     | ++++        | +++(+) | +++  | ++    |
| 12                     | ++++        | ++++   | ++++ | ++(+) |

Addition of 2% glycerol to the Dubos medium gave varying results. Sometimes glycerol potentiated slightly the inhibitory effect of E 600 (7); however, these results were not always reproducible.

The potentiating effect of Tween 80 on the inhibition of growth of tubercle bacilli by E 600 could also be shown in experiments in the medium of Proskauer and Beck. This medium normally contains no Tween, and the inhibition of growth of tubercle bacilli by 0.3 mg% E 600 was slight. When, however, Tween or Tween and albumin had been added, growth was suppressed by 0.3 mg% E 600 for at least 45 days (Table II).

In cultures of some saprophytic mycobacteria (*M. phlei* and strain S 3048 from human sputum) growing at a low temperature (22°C.), the presence of Tween was again necessary for inhibition of growth by small amounts of E 600. A concentration of 0.3 mg% E 600 had no visible effect on cultures grown in ordinary broth,

with or without the addition of 2% glycerol. When, however, Tween 80 was added to the broth, 0.3 mg% E 600 caused a marked inhibition for at least two weeks.

TABLE II.

Effect of E 600 on tubercle bacilli in the medium of Proskauer and Beck and in its modifications (37°C.).

| Days after<br>inoculation | Proskauer and Beck medium with the addition of |      |                  |     |                                    |     |
|---------------------------|------------------------------------------------|------|------------------|-----|------------------------------------|-----|
|                           | 0                                              |      | Tween 80 (0.05%) |     | Tween 80 (0.05%)<br>albumin (0.2%) |     |
|                           | concentration of E 600 (mg%)                   |      |                  |     |                                    |     |
|                           | 0                                              | 0.3  | 0                | 0.3 | 0                                  | 0.3 |
| A 3                       | +                                              | —    | +                | —   | +                                  | —   |
| 4                         | +(+)                                           | +    | ++               | —   | ++                                 | —   |
| 9                         | ++++                                           | ++   | ++++             | —   | ++++                               | —   |
| 12                        | +++++                                          | ++++ | +++++            | —   | +++++                              | —   |
| 45                        |                                                |      |                  | —   |                                    | —   |
| B 4                       | —                                              | —    | +                | —   | ++                                 | —   |
| 6                         | (+)                                            | (+)  | ++               | —   | ++—                                | —   |
| 9                         | +                                              | +    | ++++             | —   | ++++                               | —   |
| 12                        | +(+)                                           | +(+) | ++++             | —   | +++++                              | —   |
| 17                        | ++                                             | ++   | ++++             | —   | +++++                              | —   |
| 45                        |                                                |      |                  | —   |                                    | —   |

A. all flasks were inoculated with a pellicle of strain H37Rv grown on the medium of Proskauer and Beck; in those with Tween the pellicle sank immediately.

B. all flasks were inoculated with a small quantity of culture of H37Rv grown in Dubos without Tween.

TABLE III.

Effect of E 600 on tubercle bacilli of sputum on slides in the medium of Price (haemolysed human blood). Growth observed at low magnification (100 ×).

| Days after inoculation | E 600 mg% |             |           |      |
|------------------------|-----------|-------------|-----------|------|
|                        | 0         | 1           | 5         | 10   |
| 3                      | — —       | — —         | — —       | — —  |
| 5                      | + ++      | — +         | — —       | — —  |
| 7 <sup>1)</sup>        | ++++      | +(+) +(+) + | +         | — —  |
| 10                     | ++++      | ++++ +++++  | +++ ++(+) | ++ + |

<sup>1)</sup> see fig. 1.

Table III shows the effect of E 600 on the growth of tubercle bacilli in the medium of Price; concentrations above 1 mg % inhibit the growth. As in the previous experiments in the media of Dubos and of Proskauer and Beck, the inhibition of growth was reversible.

The individual bacteria lying isolated from each other on the glass slides in the beginning of the experiment grew to form colonies which generally were visible with low magnification ( $100\times$ ) after 4 days and later formed larger clumps and cords (see fig. 2). In the presence of E 600, growth of the bacteria was delayed for several days; the higher the concentration of E 600, the longer growth was suppressed. Smears which had been grown in 10 mg % E 600 showed in the end the same number of bacterial colonies as the controls (fig. 2). It is apparent, therefore, that growth of tubercle bacilli after inhibition by E 600 does not arise from a few resistant specimens but from the whole population.

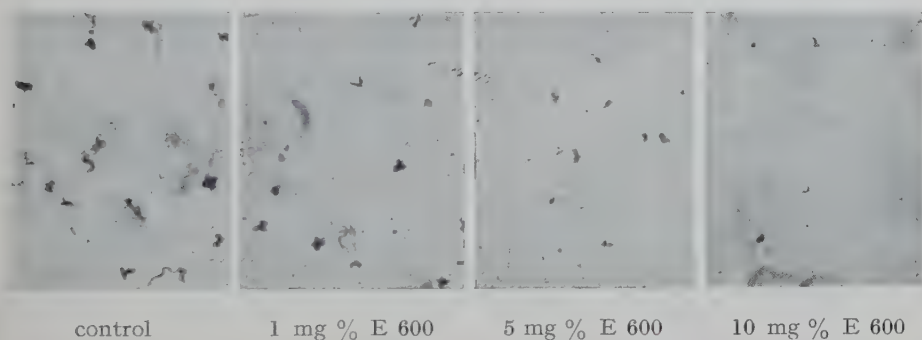


Fig. 1. Tubercle bacilli in sputum grown on glass slides in the medium of Price with varying amounts of E 600 during 7 days at  $37^{\circ}\text{C}$ . ( $100\times$ ).

## DISCUSSION.

The reversibility of the growth inhibition by E 600 is almost certainly due to destruction of this inhibitor. It has been found that E 600 is slowly hydrolysed in water, and further that the hydrolysis is catalysed by an enzyme which is designated as A-esterase (1). These findings are supported by results obtained in the present investigation. It was found that the concentration of E 600 in the medium of Dubos decreases to one-tenth of the original concentration in 5–10 days at  $37^{\circ}\text{C}$ . (7). In the medium of Proskauer and Beck, on the other hand, the concentration of E 600 decreases to one-tenth only after incubation for approximately 100 days at  $37^{\circ}\text{C}$ .

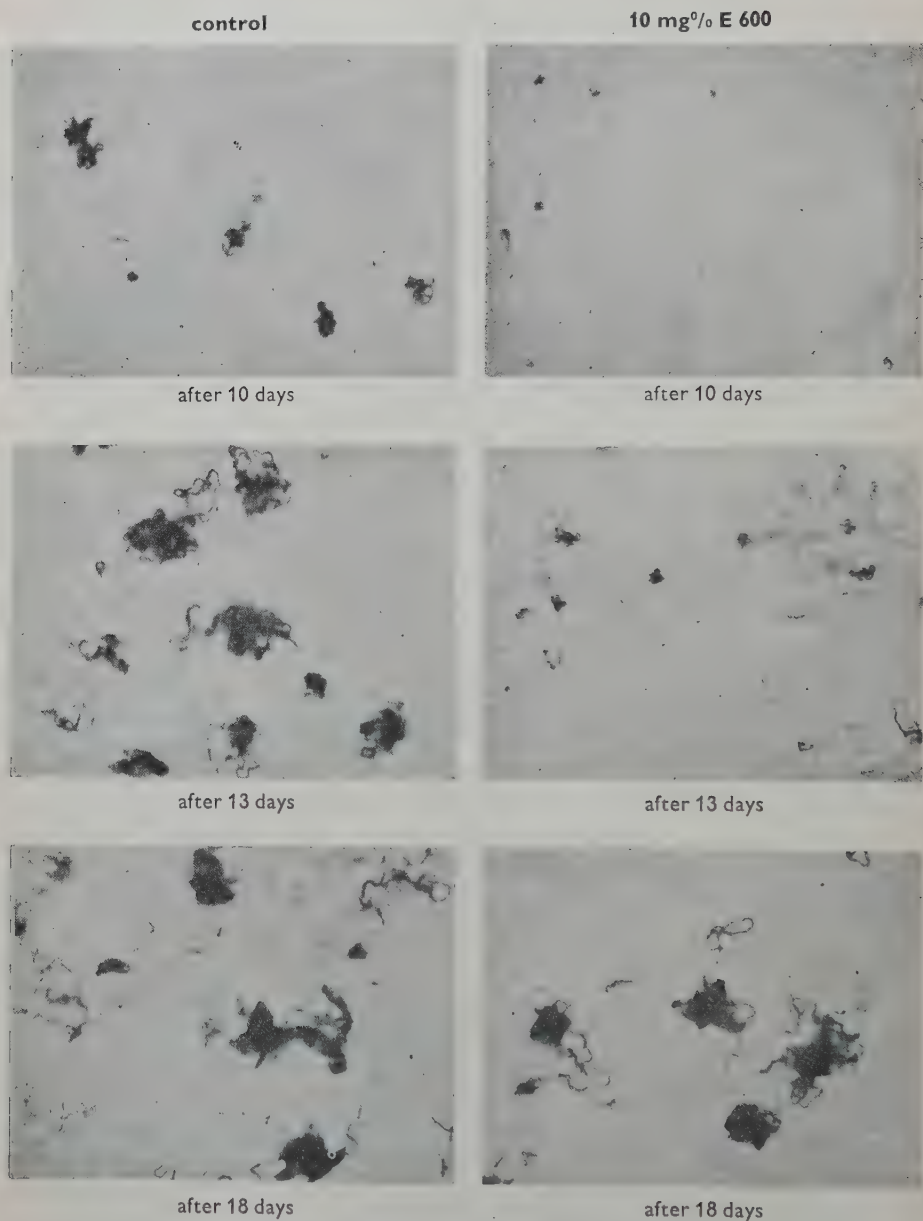


Fig. 2. Tubercle bacilli in sputum grown on glass slides in the medium of Price stained by Hallberg's method ( $100\times$ ).

This difference in the stability of E 600 in these two media appears to be due to A-esterase in the plasma albumin present in the Dubos medium; when 0.2% albumin is added to the medium of Proskauer and Beck, E 600 breaks down about as rapidly as in the usual Dubos medium (D. K. MYERS, personal communication). It is to be expected, therefore, that after the inhibitor has been destroyed the ali-esterase inhibition will also disappear and the tubercle bacilli will begin to grow again.

The presence of Tween 80 or glycerol has very little effect on the rate at which E 600 is destroyed, whether the medium contains plasma proteins or not. The potentiating effect of Tween on the inhibition of growth by E 600 must therefore have some other reason. Tween 80 is known to have a high surface activity and it seems probable that the presence of Tween is conducive to the action of E 600 by changing the permeability of the waxy substance of the mycobacteria.

A similar potentiation of the inhibitory action of penicillin and streptomycin on tubercle bacilli by Tween 80 has been observed by KIRBY and DUBOS (4) and by FISHER (3). The lower inhibitory activity of E 600 on the growth of tubercle bacilli in media which do not contain Tween could be accounted for on the basis that, in the absence of Tween, E 600 has considerable difficulty in penetrating into the mycobacteria.

However, it is apparent from the above results that Tween 80 is not essential to the inhibition of growth by E 600. A definite inhibition is seen with higher concentrations of E 600 in the medium of Price, even though E 600 deteriorates rapidly in this medium owing to the presence of A-esterase in human blood. The concentration of E 600 decreased to one-tenth in 3—4 days. Since the resumption of growth after an initial inhibition by E 600 (fig. 2) can be explained by the destruction of this inhibitor, it would be expected that a more stable inhibitor of ali-esterase would cause a longer lasting inhibition of growth.

### S u m m a r y.

The effect of the potent ali-esterase inhibitor E 600 (paraoxon) on the growth of tubercle bacilli is potentiated by the addition of Tween 80 to the medium. This may be due to a change in the permeability of the bacteria for the inhibitor.



Growth of tubercle bacilli from sputa is also inhibited by E 600 in the haemolysed human blood medium of Price. Cultures of tubercle bacilli in media with E 600 begin to grow after some time, when the inhibitor has broken down. The results obtained by the method of Price indicate that growth of the cultures does not originate from a few resistant cells but from the whole population which may have regained the ali-esterase activity essential to their growth.

### A d d e n d u m.

The ali-esterase inhibitor (E 600) used in this investigation is also a potent inhibitor of cholinesterases and therefore not suitable for *in vivo* experiments. However, after completion of this paper a number of selective ali-esterase inhibitors were synthesized by D. K. MYERS at the Pharmaco-therapeutic Laboratory, University of Amsterdam. *In vitro*, one of these compounds, a methylamino-phosphonate, was found to inhibit the ali-esterases a thousand times better than the cholinesterases. This finding suggests that it may eventually be possible to inhibit the growth of tubercle bacilli *in vivo* with ali-esterase inhibitors.

### A c k n o w l e d g m e n t s.

The author is greatly indebted to Miss LOUWERSE for her help with the cultures of tubercle bacilli and to Mr. D. K. MYERS for determining the concentrations of E 600 in the course of the various experiments.

### R e f e r e n c e s.

1. ALDRIDGE, W. N. 1953. *Biochem. J.* **53**, 117.
2. DUBOS, R. J. and DAVIS, B. D. 1946. *J. Exp. Med.* **83**, 409.
3. FISHER, M. W. 1948. *Am. Rev. Tuberc.* **57**, 58.
4. KIRBY, W. M. M. and DUBOS, R. J. 1947. *Proc. Soc. Exp. Biol. Med.* **66**, 120.
5. MENDEL, B. and MORTIMER, E. 1944. M.P. 36 Report to National Research Council of Canada.
6. MENDEL, B. and MYERS, D. K. 1953. *Biochem. J.* **53**, XVI.
7. MENDEL, B., MYERS, D. K., UYLDERT, I. E., RUYS, A. C. and DE BRUYN, W. M. 1953. *Brit. J. Pharmacol.* **8**, 217.
8. MYERS, D. K. and MENDEL, B. 1953. *Biochem. J.* **53**, 16.
9. MYERS, D. K., SIMONS, S. P. and SIMONS, E. G. L. 1953. *Biochem. J.* in press.
10. PRICE, D. M. 1941. *J. Path. Bact.* **53**, 327.

(Netherlands Institute for Dairy Research, Hoorn).

## ACID PRODUCTION BY *Sc. THERMOPHILUS*

by

**H. LOLKEMA**<sup>1)</sup>

(Received May 5, 1953).

### 1. INTRODUCTION.

In the course of experiments made with the yoghurt-bacteria, *Lb. bulgaricus* and *Sc. thermophilus*, it was found desirable to calculate the acid production per cell per hour of *Sc. thermophilus*, when cultivating this bacterium in a pure culture and in a mixed culture.

The fact is, that in a mixed culture the development of *Sc. thermophilus* is stimulated, owing to the amino acids, which are freed from the milk-proteins by *Lb. bulgaricus* (PETTE and LOLKEMA, 1950*b*). This stimulating action results finally in a greater number of cells and in an increased acid production (PETTE and LOLKEMA, 1950*a*).

This increased acid production may possibly be caused by the mere fact of a greater number of cells, but it might be conceived, that the acid production per cell per hour is also increased by the stimulating action of the amino acids. It will be seen from the following calculation, that the two phenomena are related.

### 2. PURPOSE OF THE CALCULATION.

In order to trace the influence of the amino acids on the acid production per cell per hour it is necessary to calculate the amount of the latter.

In doing so we are faced with the difficulty, that this quantity is not a constant one, but depends upon the physiological age of the culture. When comparing different cultures it is necessary to adopt a standard for this physiological age.

---

<sup>1)</sup> The author is now Director of the Government Dairy College at Bolsward.

In our calculation we chose for this the generation time ( $= g$ ), and the object was to determine both in the pure culture and in the mixed culture, the relation between the generation time and the acid production per cell per hour.

The experiments have proved, that the major part of the acid is produced in the phase of the diminishing growth rate. In literature no method was encountered giving accurate calculations for this phase.

The following calculation is based on the idea formulated as the Law of the Diminishing Returns (VAN UVEN, 1935).

### 3a. METHOD OF CALCULATION OF THE NUMBER OF BACTERIA.

Determining the number of bacteria in a "growing" culture we learn, that the rate of multiplication is very slow immediately after the inoculation. For some reason or other the bacteria need time to start.

After this lag phase the bacteria keep dividing at optimum rate for some time. For this phase we can assume that at some moment the increase of the number of bacteria per unit of time is directly proportional to the number of bacteria present at that moment, thus:

$$\frac{dN_t}{dt} = cN_t \quad (\text{I})$$

or:

$$\frac{d \log N_t}{dt} = C_1 \quad (\text{II})$$

From the equation (II) it can easily be seen that the increase of the logarithm of the number of bacteria per unit of time is constant. This is why this phase is called the logarithmic growth phase.

After some time the bacteria are checked in their development. The equation (II) is then no longer valid, but wants correction. Analogous to the Law of the Diminishing Returns the following formula is chosen:

$$\frac{d \log N_t}{dt} = h (Q - \log N_t) \quad (\text{III})$$

In this formula  $h$  is constant and  $Q$  is the logarithm of the number of bacteria to be obtained asymptotically, a quantity, which we, theoretically, keep approaching, without reaching it, thus  $Q = \log N_\infty$ .

The solution of the equation (III) gives (VAN UVEN, 1935):

$$\log N_t = Q - H \cdot 10^m \times t, \quad (\text{IV})$$

In this formula  $m$  ( $= -Mh$  and  $M = 0.43$ ) and  $H$  are constant values.

The values for  $Q$ ,  $H$  and  $m$  can be found from the experimental data, since:

$$\begin{aligned} Q - \log N_t &= H \cdot 10^m \times t, \\ \log (Q - \log N_t) &= m \times t + \log H \end{aligned} \quad (\text{V})$$

We should therefore find the value of  $Q$ , which with the experimental data in the equation V gives the best approach to the equation of a straight line.

Drawing the line gives the constant values  $m$  and  $H$ , resp. by determining the gradient and the value of  $(Q - \log N_t)$  for  $t = 0$ .

With the aid of the constant values for  $Q$ ,  $m$  and  $H$  it is possible to calculate the number of bacteria at any moment.

### 3b. CALCULATION OF THE GENERATION TIME.

As has been stated, our object is to determine the relation between the generation time and the acid production per cell per hour.

The generation time at any given moment can be found from the equation of the tangent on the curve, giving the relation between  $\log N$  and  $t$ .

If we assume for moment  $t$ , that the cells are dividing as in the logarithmic phase, with generation time  $g_t$ , then the growth curve would continue in accordance with the tangent of the curve for the moment  $t$ .

For the logarithmic growth phase can be obtained:

$$N_{t+1} = N_t \cdot \frac{1}{2^{g_t}},$$

in which equation:

$$\begin{aligned} N_{t+1} &= \text{number of cells for moment } t + 1 \text{ (hours);} \\ N_t &= \text{,, ,, ,, ,, } t \text{ (,, );} \\ g_t &= \text{generation time for moment } t. \end{aligned}$$

From this equation we can derive equation VI:

$$g_t = \frac{\log 2}{\log N_{t+1} - \log N_t}$$

or:

$$g_t = \frac{0.30103}{\frac{d \log N_t}{d_t}} \quad (\text{VI})$$

For  $\frac{d \log N_t}{d_t}$  we may write (equation III):

$$\frac{d \log N_t}{d_t} = h (Q - \log N_t) \text{ in which } h = -\frac{m}{0.43}.$$

In this way we get for  $g_t$ :

$$g_t = \frac{0.30103}{-\frac{m}{0.43} (Q - \log N_t)} \quad (\text{VI}^1)$$

This formula makes it possible to calculate the generation time at any moment.

#### 4. CALCULATION OF THE ACID PRODUCTION PER CELL PER HOUR.

As we have explained above (page 154) our object is to calculate both the generation time and the acid production per cell per hour at the same moment.

Supposing we have  $N$  cells with a constant acid production ( $= a$ ) then it is possible to express the quantity of acid produced for  $b$  hours in the formula:

$$Z = N \times a \times b.$$

In actual experience the above mentioned conditions are not fulfilled, but if instead of  $b$  hours we take one minute, one second or less,  $N$  and  $a$  are practically constant. The calculated quantity  $\Delta Z$  is now:

$$\Delta Z = N \times a \times \Delta t,$$

or:

$$\frac{dZ}{dt} = N \times a$$

or:

$$a = \frac{\frac{dZ}{dt}}{N}$$

or:

$$a_t = \frac{\frac{dZ}{dt}}{N_t} \quad (\text{VII})$$

A knowledge of the formula for the acid production in connection



with the time, enables us to calculate the acid production per cell per hour.

This formula is often partly the formula for a straight line ( $y = mx + q$ ) and partly of the type as given by the formula IV (the latter formula holds of course only when the rise of the acidity is no longer proportional to the time).

With the aid of the formulae IV, VI<sup>1</sup> and VII we are able to calculate for each moment:

- a. the generation time of the bacteria;
- b. the acid production per cell per hour.

The relation between these two quantities can also be determined.

## 5. PERFORMING THE CALCULATIONS.

The calculations, on the principles mentioned, were made for cultures of *Sc. thermophilus*, with and without addition of stimulating agents (PETTE and LOKEMA, 1950b).

The following example is the treatment of the data of an experiment made with *Sc. thermophilus* W<sub>s</sub>, activated with a sterile filtrate of a milk culture of *Lb. bulgaricus* IIIB. The percentage of inoculation was 1 and 10% of the filtrate was added.

In this culture the growth curve of the diplococci was found by the Breed-count, and the acid production by titration of 20 g of the culture. Table 1 gives the results.

TABLE 1.

Number of diplococci and acid production in the activated culture W<sub>s</sub>.

| time<br>(hours) | $N \times 10^{-6}$ | $\log N$ | acidity<br>ml n/10/20 g |
|-----------------|--------------------|----------|-------------------------|
| 9.30            | 5                  | 6.70     | 3.8                     |
| 10.30           | 15                 | 7.18     | 4.0                     |
| 11.30           | 210                | 8.32     | 5.3                     |
| 12.30           | 580                | 8.76     | 8.0                     |
| 13.30           | 840                | 8.92     | 9.8                     |
| 14.00           | —                  | —        | 10.4                    |
| 14.30           | 930                | 8.97     | 10.9                    |
| 15.30           | 950                | 8.98     | 11.5                    |

Figure 1 was made from table 1. From this figure it can easily be seen, that but little acid is produced at the moment the growth

curve bends (time 11.30 h). When the cultures are not activated this bend is found much earlier.

The following calculation only bears on the period after 11.30 h.

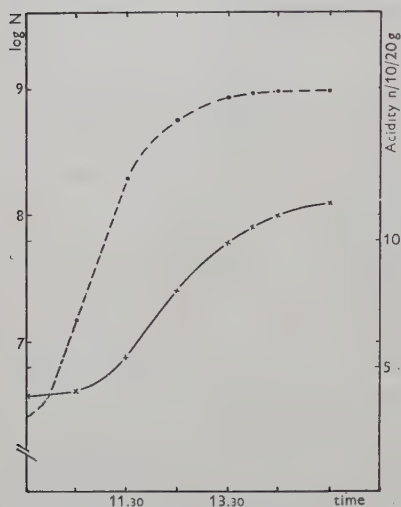


Fig. 1. Development of the number of bacteria (----) and of the acid production (—) in an activated culture  $W_s$ .

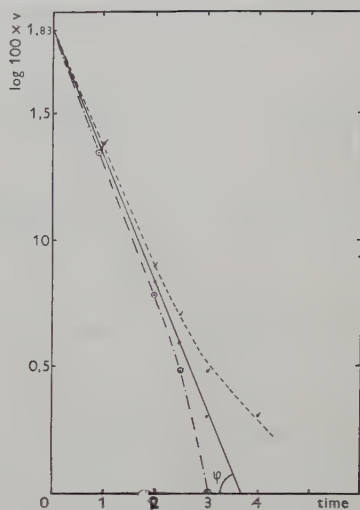


Fig. 2. Relation between  $v(Q - \log N_t)$  and time (see table 2).

#### a. Determination of the formula for the growth curve.

For this purpose we chose for the maximum value of  $\log N$  ( $= Q$  in the formulae IV and V) successively: 8.98; 8.99 and 9.00.

The differences existing between these values and the data of the experiment,  $v$  (multiplied by 100 for the sake of convenience), were ascertained and the logarithm of these differences were plotted graphically in relation to the time (table 2 and fig. 2).

TABLE 2.  
Logarithm  $100 \times (Q - \log N_t)$  for different values of  $Q$ .

| time<br>(hours) | $t$            | $V = Q - \log N_t$ |            |            | $\log 100 \times v$ |            |            |
|-----------------|----------------|--------------------|------------|------------|---------------------|------------|------------|
|                 |                | $Q = 8.98$         | $Q = 8.99$ | $Q = 9.00$ | $Q = 8.98$          | $Q = 8.99$ | $Q = 9.00$ |
| 11.30           | 0              | 0.66               | 0.67       | 0.68       | 1.83                | 1.83       | 1.83       |
| 12.30           | 1              | 0.22               | 0.23       | 0.24       | 1.34                | 1.36       | 1.38       |
| 13.30           | 2              | 0.06               | 0.07       | 0.08       | 0.78                | 0.85       | 0.90       |
| 14.00           | $2\frac{1}{2}$ | 0.03               | 0.04       | 0.05       | 0.48                | 0.60       | 0.70       |
| 14.30           | 3              | 0.01               | 0.02       | 0.03       | 0.00                | 0.30       | 0.48       |
| 15.30           | 4              | 0.00               | 0.01       | 0.02       | 0.00                | 0.00       | 0.30       |

The value 8.99 thus procures differences which give a linear relation with the time. Hence the maximum value for  $\log N_t (= Q)$  is 8.99. The rectilinear adjustment gives the following equation:

$$\log 100 \times v = m \times t + C,$$

in which

$$m = \frac{t g \cdot \varphi}{3.65} = -\frac{1.83}{3.65} = -0.5$$

and

$$C = 1.83$$

Thus it is possible to give  $\log N_t$  as a function of  $t$ :

$$\begin{aligned} 2 + \log v &= -0.5 \times t + 1.83 \\ 2 + \log (Q - \log N_t) &= -0.5 \times t + 1.83 \\ \log (8.99 - \log N_t) &= -0.5 \times t - 0.17 \\ 8.99 - \log N_t &= 10^{-0.5 \times t} \times 10^{-0.17} \\ 8.99 - \log N_t &= 0.67 \times 10^{-0.5 \times t} \\ \log N_t &= 8.99 - 0.67 \times 10^{-0.5 \times t} \end{aligned} \quad (IV^1)$$

This formula gives us the number of bacteria at any moment.

#### b. Calculation of the generation time.

In the formula VI<sup>1</sup> we found:

$$g_t = \frac{0.30103}{-\frac{m}{0.43} (Q - \log N_t)}$$

hence

$$g_t = \frac{0.30103}{-\frac{m}{0.43} \times 0.67 \times 10^{-0.5 \times t}}$$

or

$$g_t = \frac{0.30103}{0.78 \times 10^{-0.5 \times t}} \quad (VI^2)$$

#### c. Calculation of the acid production per cell per hour.

As the graph for the relation of acidity and time after 11.30 h shows the same course as the growth curve, the same method of calculation was also used. The result is:

$$A(\text{cidity}) = 12.5 - 7.2 \times 10^{-0.22 \times t},$$

The acidity is expressed in ml n/10 alkali per 20 g of the culture, while again  $t = 0$  for the time 11.30 hrs.

The increase of the acidity per unit of time is given by the

differential quotient (calculated as shown in formula III<sup>1</sup>):

$$\frac{dA}{dt} = \frac{0.22}{0.43} \times 7.2 \times 10^{-0.22 \times t} = 3.6 \times 10^{-0.22 \times t}.$$

When calculated in mg of lactic acid per g of culture this gives:

$$\frac{dA}{dt} = \frac{1}{20} \times 9 \times 3.6 \times 10^{-0.22 \times t} = 1.62 \times 10^{-0.22 \times t}.$$

For the acid production per cell per hour, in mg, at the moment  $t$  we consequently get the formula (according to formula VII):

$$a_t = \frac{1.62 \times 10^{-0.22 \times t}}{N_t}. \quad (\text{VII}^1)$$

Thus the formulae VI<sup>2</sup> and VII<sup>1</sup> give the values for the generation time and the quantity of acid production per cell per hour at the same moment.

For the given data we get (table 3):

TABLE 3.  
Calculation of  $g_t$  and  $a_t$  from the formulae VI<sup>2</sup>, IV<sup>1</sup>, VII<sup>1</sup>.

| moment                | 11.30 | 11.45 | 12.00 | 12.30 | 13.30 | 14.30 |                  |
|-----------------------|-------|-------|-------|-------|-------|-------|------------------|
| $t$                   | 0     | 0,25  | 0,5   | 1     | 2     | 3     |                  |
| $N_t$                 | 210   | 310   | 410   | 600   | 840   | 930   | $\times 10^{-6}$ |
| $\frac{d \log N}{dt}$ | 0.78  | 0.59  | 0.44  | 0.26  | 0.08  | 0.025 |                  |
| $\frac{dZ}{dt}$       | 1.62  | 1.44  | 1.26  | 0.99  | 0.59  | 0.36  | $\text{‰}$       |
| $g_t$                 | 0.38  | 0.51  | 0.70  | 1.10  | 3.75  | 12    | hours            |
| $a_t$                 | 77    | 47    | 30    | 16    | 7     | 4     | $10^{-10}$ mg    |

## 6. APPLICATIONS OF THE TREATMENT.

A. As stated in the introduction, the motive for the mathematical treatment of the results was to find out whether the stimulating action of *Lb. bulgaricus* on *Sc. thermophilus* only causes a greater number of cells or gives rise to an increased acid production per cell per hour as well.

It is further stated, that this latter magnitude depends upon the physiological age of the culture. This normal change must be eliminated, which is done by taking the generation time as standard of comparison.

If in both cultures of *Sc. thermophilus* we find the same relation between  $a$  (= acid production per cell per hour) and  $g$  (generation time) we may draw the conclusion, that the stimulating action of the amino acids does not superinduce the acid production.

By means of the calculations described, the relation between  $a$  and  $g$  was ascertained for a few species of *Sc. thermophilus*, with and without the stimulating action of sterile filtrates of cultures in milk of *Lb. bulgaricus* species.

Fig. 3 shows the result of some of these observations with *Sc. thermophilus* W<sub>s</sub>. In this graph the dots indicate the values calculated for the activated cultures and the circles those for the non-activated cultures. It is clearly seen, that in both cases the same relation is found between  $a$  and  $g$ . So we may draw the conclusion that the stimulation primarily affects the development of the cells.

Fig. 3 also shows the phenomenon, that a short generation time is attended with a high acid production per cell per hour. In a mixed culture (or in an activated culture) the generation time is short for a long period (PETTE and LOLKEMA, 1950*a,b*). This also implies a high acid production per cell per hour for a long period. The average acid production per cell per hour in the course of the first period will also be higher than in an untreated culture.

Hence the rapid acidification of a yoghurt-culture is caused by the rapid multiplication of the bacteria in the first stage. This results in the production of a great number of cells in a short period, whilst the average acid production per cell per hour is high, as is shown in the preceding lines.

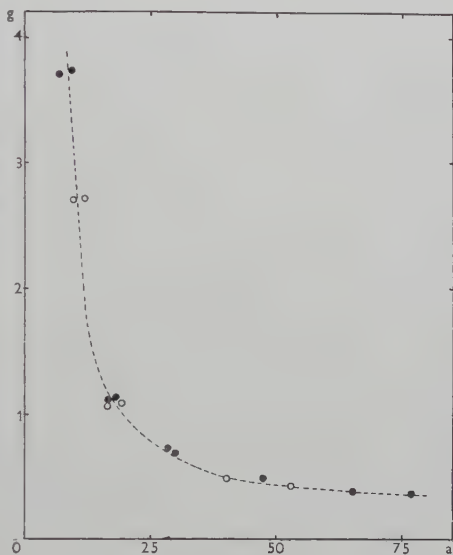


Fig. 3. Relation between acid production (a) per cell per hour and generation time (b) in cultures of *Sc. thermophilus* W<sub>s</sub>  
● activated. ○ not activated



**B.** Calculation of the acid production from the rods and the cocci in a yoghurt-culture.

To ascertain the way the acid production in a yoghurt-culture takes place, the inverse calculation was made.

The example here given shows the acidification in the yoghurt

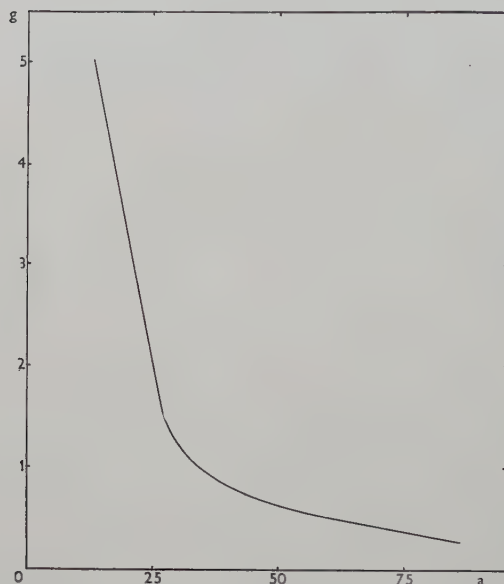


Fig. 4. Relation between generation time ( $g$ ) and acid production ( $a$ ) per cell per hour in cultures of *Sc. thermophilus* St<sub>s</sub>

IS<sub>t</sub>, composed of *Lb. bulgaricus* I<sub>b</sub> and *Sc. thermophilus* St<sub>s</sub>. The relation between  $a$  and  $g$  was previously determined by means of a great number of separate tests (Fig. 4). To calculate the acid production in the course of half an hour it was assumed, that during this period the bacteria increased as in the logarithmic phase. Hence it is possible to use the formulae for this phase.

We have (PORTER, 1946; SUPINSKA and MATUSZEWSKI, 1936):

$$g = \frac{t \log 2}{\log b - \log B} \text{ in which:} \quad (1)$$

$g$  = generation time in hours;

$t$  = time in hours;

$B$  = number of bacteria at moment 0;

$b$  = number of bacteria at moment  $t$ .

$$k = \frac{0.692}{g} \text{ in which:} \quad (2)$$

$k$  = velocity-coefficient;

$g$  = generation time in hours.

$$a = \frac{k \times Z}{b - B} \text{ in which:} \quad (3)$$

$a$  = acid production per cell per hour in mg;

$Z$  = increase of the acidity in ‰.

$$Z = \frac{a(b - B)}{k} \quad (3')$$

With this latter formula the acid production for a fixed period can be calculated if  $a$ ,  $b$ ,  $B$  and  $k$  are known.

We find  $b$  and  $B$  by counting the number of bacteria according to the Breed-method. Next we calculate  $g$  (formula 1) and also  $k$  (formula 2). In Fig. 4 we find for the calculated  $g$  the corresponding  $a$ .

In a related test the following data for the culture IS<sub>t</sub> were found (table 4):

TABLE 4.

Data for the calculation of the acid production by the rods and the diplococci in the yoghurt IS<sub>t</sub>.

| time<br>(hours) | acidity<br>ml n/10/20 g | rods<br>× 10 <sup>-6</sup> | log. number<br>of rods | diplococci<br>× 10 <sup>-6</sup> | log. number<br>of diplococci |
|-----------------|-------------------------|----------------------------|------------------------|----------------------------------|------------------------------|
| 0               | 3.8                     | 9.2                        | 6.96                   | 11.3                             | 7.05                         |
| $\frac{1}{2}$   | 4.2                     | 13                         | 7.11                   | 55                               | 7.74                         |
| 1               | 5.4                     | 37                         | 7.57                   | 200                              | 8.30                         |
| $1\frac{1}{2}$  | 7.6                     | 86                         | 7.93                   | 440                              | 8.64                         |
| 2               | 11.0                    | 170                        | 8.23                   | 480                              | 8.68                         |
| $2\frac{1}{2}$  | 13.3                    | 230                        | 8.36                   | 560                              | 8.75                         |
| 3               | 15.3                    | 310                        | 8.49                   | 570                              | 8.76                         |
| $3\frac{1}{2}$  | 16.6                    | 350                        | 8.54                   | 500                              | 8.70                         |

TABLE 5.

Calculation of the acid production by the diplococci in yoghurt IS<sub>t</sub>.

| time<br>(hours) | log $B$<br>(graph<br>5) | log $b$ —<br>log $B$ | $g$<br>form.<br>(1) | $k =$<br>$\frac{0.692}{g}$ | $a$<br>(graph<br>4) | $(b-B)$<br>× 10 <sup>-6</sup> | $Z$<br>‰ | $Z$<br>ml n/10<br>per 20 g |
|-----------------|-------------------------|----------------------|---------------------|----------------------------|---------------------|-------------------------------|----------|----------------------------|
| 0               | 7.05                    | —                    | —                   | —                          | —                   | —                             | —        | —                          |
| $\frac{1}{2}$   | 7.74                    | 0.69                 | 0.22                | 3.15                       | 100                 | 44                            | 0.14     | 0.31                       |
| 1               | 8.30                    | 0.56                 | 0.27                | 2.56                       | 90                  | 145                           | 0.51     | 1.13                       |
| $1\frac{1}{2}$  | 8.60                    | 0.30                 | 0.50                | 1.38                       | 60                  | 198                           | 0.93     | 2.08                       |
| 2               | 8.71                    | 0.11                 | 1.36                | 0.51                       | 29                  | 115                           | 0.65     | 1.44                       |
| $2\frac{1}{2}$  | 8.75                    | 0.04                 | 3.75                | 0.18                       | 18                  | 49                            | 0.49     | 1.09                       |
| 3               | 8.76                    | 0.01                 | 15.00               | 0.05                       | 4?                  | 13                            | 0.10     | 0.22                       |
| $3\frac{1}{2}$  | 8.76                    | —                    | —                   | —                          | —                   | —                             | —        | —                          |

From these data we made Fig. 5. As the counting of bacteria is not very accurate the numbers of bacteria given in Fig. 5 were used for the calculation.

This calculation was made for intervals of half an hour as stated in table 5.

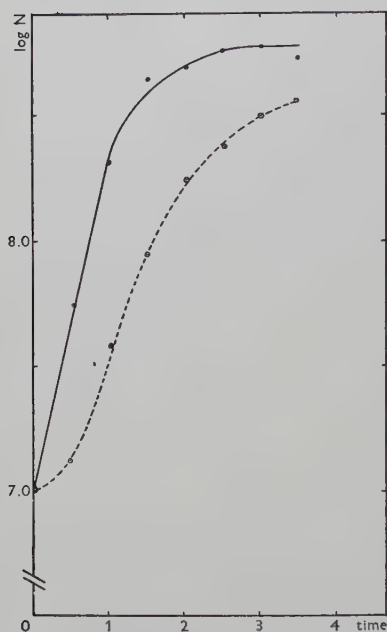


Fig. 5. Development of the number of bacteria in the yoghurt-culture  
 $IS_t$  ————— diplococci,  
 - - - - - rods.

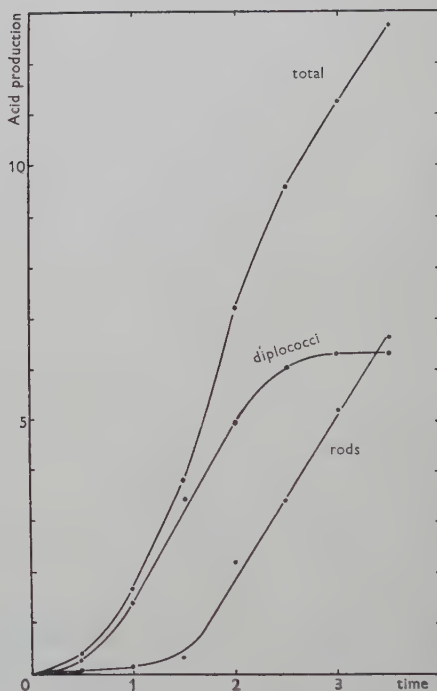


Fig. 6. Acid production in yoghurt  $IS_t$

When we add up the acid productions of the intervals, we are in a position to calculate the quantity of acid produced by the cocci for a fixed space of time.

The difference between the total acidity (found by titration) and the acid production calculated for the diplococci is the acid production by the rods. We find the following quantities (table 6).

These results are plotted in Fig. 6. It is clearly shown, that acidification in yoghurt in the first stage is caused by the activity of *Sc. thermophilus*. However the growth of these bacteria is soon checked for they are rather sensitive to acid.

TABLE 6.  
Acid production by diplococci and rods in yoghurt IS<sub>t</sub>.

| time<br>(hours) | total acid<br>production | acid produc-<br>tion of St <sub>s</sub> | acid produc-<br>tion of I <sub>b</sub> |
|-----------------|--------------------------|-----------------------------------------|----------------------------------------|
| 0               | —                        | —                                       | —                                      |
| $\frac{1}{2}$   | 0.4                      | 0.31                                    | 0.09                                   |
| 1               | 1.6                      | 1.44                                    | 0.16                                   |
| $1\frac{1}{2}$  | 3.8                      | 3.52                                    | 0.28                                   |
| 2               | 7.2                      | 4.96                                    | 2.24                                   |
| $2\frac{1}{2}$  | 9.5                      | 6.05                                    | 3.45                                   |
| 3               | 11.5                     | 6.27                                    | 5.23                                   |
| $3\frac{1}{2}$  | 12.8                     | 6.27                                    | 6.53                                   |

Even at this moment the rods still form the acid in great quantities. Accordingly the second stage of the acidification is caused by the rods.

### S u m m a r y.

The calculation based on the idea formulated as the Law of Diminishing Returns has here been applied to the development of *Sc. thermophilus* in milk-cultures during the phase of diminishing growth rate. The values calculated correspond fairly well with the data found in the experiment.

An application of this method is given in the calculation of the relation between the acid production per cell (diplococci) per hour and the generation time. It is shown, that a quick multiplication corresponds to a high acid production and inversely a long generation time gives a small acid production.

The relation between these quantities enables us to account for the rapid acidification in yoghurt. It also enables us to calculate the acid production in yoghurt by the cocci and the rods from the experimental data.

By making this calculation we could prove that in the first stage the cocci are the acid producers. In the second stage the rods keep producing acid whereas the growth of cocci is checked by the lactic acid which has then for the greater part been produced by themselves.

We wish to express our indebtedness to dr J. W. PETTE, director of the Netherlands Institute for Dairy Research, for his stimulating interest in this study and to prof. dr M. VAN UVEN and prof. dr N. H. KUIPER of Wageningen for reading and correcting the manuscript.

## Literature.

- PETTE, J. W. and LÖLKEMA, H. 1950*a*. *Neth. Milk and Dairy J.* **4**, 197.  
PETTE, J. W. and LÖLKEMA, H. 1950*b*. *Neth. Milk and Dairy J.* **4**, 209.  
PORTER, J. R. 1946. *Bacterial Chemistry and Physiology*. p. 122—124.  
SUPINSKA, J. und MATUSZEWSKI, T. 1936. *Zentr. f. Bakt.* II, **94**, 499.  
VAN UVEN, M. J. 1935. The mathematical treatment of the results of agricultural and other experiments. Groningen.
-



(From the Serum- and Vaccine-Laboratory of the N.V. Philips-Roxane,  
Weesp, Holland).

## STERILITY TEST ON WATER OIL EMULSIONS

by

**E. HERTZBERGER**

(Received July 16, 1953).

Immunisation against infectious diseases with the aid of adjuvants is a common procedure. SALK (3) published his results with influenza-virus-vaccination with adjuvants in which he used as adjuvant a normal influenza-virus-vaccine, emulsified in mineral oil. These emulsions, which are prepared by emulsifying the water-phase with the aid of a "span" type emulsifier (Arlacel A, i.e. mannide mono-oleate) in mineral oil (Bayol F), are not yet in use in general practice for several reasons. It was, however, deemed necessary to investigate in what way the sterility of such an emulsion can be controlled. The emulsion is prepared by mixing 10 parts of the water-phase (a vaccine) with 9 parts Bayol F and 1 part Arlacel A. By vigorous mixing (with a vibro-mix or by aspiration and squirting out with a syringe) a stable emulsion can be obtained which behaves as an oil, i.e. it can be mixed with any amount of oil and cannot be mixed with water. An emulsion of this kind may be made from two sterile liquids and still become infected, e.g. whilst making the emulsion or during the filling of small vials; anyhow it is necessary to have a method at hand which can demonstrate viable organisms in these emulsions with the same degree of sensitivity as is the case for aqueous suspensions or solutions.

There are some publications on the sterility of technical oils (1, 2) but the problem has never been investigated on a quantitative basis. In testing the sterility of water/oil emulsions there are two methods of obtaining results.

Assuming that the micro-organisms are evenly distributed throughout the water-phase and the oil-phase, it should be possible to make the organisms enter the surrounding water (the sterility medium) by breaking down the emulsion. The organisms will then

prove their presence by growing in the sterility medium after incubation for a definite period at 37°C. or 22°C. This breaking down of the emulsion can be attained by lightly shaking the tube with the sterility medium and the amount of emulsion to be tested. This is even easier if the sterility medium contains some emulsifier of the "tween" type, i.e. an emulsifying substance which emulsifies the oil in the water. A reversal of the original emulsion to an emulsion of oil/water type cannot be obtained in this way, because an ordinary cotton-plugged culture tube permits only a very slight shaking. Only the micro-organisms present in the water-phase will have the opportunity to grow. This is a varying percentage of the total number of micro-organisms present.

The second method is to emulsify the oil in the water. This can be arrived at by shaking vigorously. If this shaking is performed in a watery medium, containing "tween", the obtained emulsion is practically stable, without the "tween" the emulsion will be labile, an oily layer appearing on the surface after a short time. Both principles were investigated separately and combined.

#### MATERIALS AND METHODS.

A suspension of spores of *Bacillus subtilis* in 0.9% saline solution was made in an amount, sufficient for all experiments. The sterility medium used was the ordinary Tarozzi medium, giving aerobic and anaerobic micro-organisms ample opportunity to multiply. The medium was prepared in one batch. This batch was divided in three equal portions, one portion was used without any additions, to one portion 0.2% tween 80 and to the third portion 0.5% tween 80 was added (tween 80 is a poly-oxy-alkylen-derivate of sorbitan-mono-oleate). The media were dispensed in tubes (9 ml/tube) and the usual three pieces of meat were added. All tubes were incubated for 6 days at 37°C. before use (sterility control). In the experiments where shaking was omitted, the tubes were cotton plugged in the normal way. For the shaking experiments the tubes were closed with rubber bungs. The latter were separately sterilised by boiling and were put on the tubes with sterile precautions after these were supplied with the emulsion to be tested. A shaking machine was used like that employed for the Kahn reaction and the shaking time was always maintained at exactly 5 minutes. The contents of the tube were shaken in the range between the bottom and the rubber bung.

The "Salk" emulsion was prepared in a rubber-capped vial. The spore suspension was mixed with adjuvant (10 parts spore suspension with 9 parts Bayol F and 1 part Arlacel A). This adjuvant mixture was sterilised beforehand in a closed vial by heating at 160°C. for one hour. With the aid of a sterile 10 ml all-glass syringe, fitted with a medium calibre needle, the two components were homogenized by drawing them up and down in the syringe. The control spore suspension consisted of one part spore suspension and one part 0.9% saline solution.

For the determination of the number of demonstrable spores, use was made of the tables of M(ost) P(robable) N(umber) as used in the normal water-analysis of coliform organisms (4). The amount of material introduced however was 1/10 of the amount described in these tables, the series consisting of 5 tubes to which were added 1 ml, 5 tubes with 0.1 ml, and 5 tubes with 0.01 ml. The amounts of 1 ml and 0.1 ml were measured with a graduated 1 ml pipette and the amounts of 0.01 ml with a tuberculin syringe fitted with a fine calibre needle. After inoculation the tubes were kept in the incubator at 37°C. for 72 hours. Presence or absence of infection with *Bacillus subtilis* was determined without the use of a microscope. Growth was assumed to have taken place if a colourless pellicle with a varying degree of clouding of the sterility medium was present. All results are given in M.P.N./ml.

#### EXPERIMENTAL.

A preliminary determination of the number of spores of *B. subtilis* present in the spore suspension was made by using counting plates in descending decimal dilutions. To exclude the presence of vegetative forms of the bacteria, the suspension was heated beforehand for 10 minutes at 80°C. The result of two series of counting plates van  $45 \times 10^6$  and  $28 \times 10^6$  viable spores/ml. So dilutions of the spore suspension for the emulsions of  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-7}$  were decided upon. For the control spore suspension one volume of 0.9% saline solution was mixed with one volume of spore suspension, diluted to  $10^{-7}$ . For the results of the test without shaking see table I.

TABLE I.

| Dilution of spore suspension         | Tarozi |          | M.P.N.            | Tarozi + 0.2% tween 80 |          | M.P.N.             | Tarozi + 0.5% tween 80 |          | M.P.N.             |
|--------------------------------------|--------|----------|-------------------|------------------------|----------|--------------------|------------------------|----------|--------------------|
|                                      | 1      | 0.1 0.01 |                   | 1                      | 0.1 0.01 |                    | 0                      | 0.1 0.01 |                    |
| $10^{-7}$ + 0.9% saline solution     | 5      | 3 0      | $158 \times 10^6$ | 5                      | 1 0      | $66 \times 10^6$   | 5                      | 0 0      | $46 \times 10^6$   |
| $10^{-7}$ + Salk adjuvant            | 0      | 1 0      | $3.6 \times 10^6$ | 1                      | 0 0      | $4 \times 10^6$    | 1                      | 1 0      | $8 \times 10^6$    |
| $10^{-6}$ + Salk adjuvant            | 0      | 5 2      | $2.6 \times 10^6$ | 0                      | 3 1      | $1.48 \times 10^6$ | 0                      | 0 1      | $0.36 \times 10^6$ |
| $10^{-5}$ + Salk adjuvant            | 0      | 5 3      | $0.3 \times 10^6$ | 0                      | 4 3      | $0.26 \times 10^6$ | 0                      | 3 4      | $0.26 \times 10^6$ |
| 0.9% saline solution + Salk adjuvant | 0      | 0 0      |                   | 0                      | 0 0      |                    | 0                      | 0 0      |                    |

The results of table I show that the number of spores, demonstrated in the emulsion, lags considerably behind the number of spores that should be present, according to the result of the control. Every concentration of emulsified spore suspension shows this phenomenon. When reading the table in a vertical direction, this shows clearly. Reading the first horizontal row (the control) it can be seen that the addition of tween 80 possibly has a slight inhibiting effect on the culture results. The rows for the emulsified spore suspension show quite clearly that the addition of tween 80 to the sterility medium does not improve the results. The light shaking movement might not completely break down the emulsion or a very large proportion of the spores might occur in the oily phase of the emulsion. The sequence of the infected tubes with the emulsified spore suspension is rather unexpected. The exclusion of the air-oxygen by the oily layer on top of the medium (especially with the 1 ml inoculation) might hamper the multiplication of the spores of *B. subtilis*. This possibility could be excluded however by inoculating a number of tubes with the control suspension and covering the medium after inoculation with a layer of sterile liquid paraffin-oil. This exclusion of the air-oxygen did not result in a diminished number of infected tubes. It is more likely that the amount of 1 ml emulsion cannot be broken up by the light shaking movement, whereas the amounts of 0.1 and 0.01 ml are small enough to allow this to occur. There still remains the possibility that the largest number of spores enters the oil-phase during the original emulsifying procedure.

To investigate the influence of shaking the emulsion in the sterility medium, the test was repeated, not using the dilutions  $10^{-6}$  and  $10^{-5}$  with the emulsions. Half of the tubes were shaken for 5 minutes in a Kahn shaking machine (Table II).

When read in a vertical direction, table II shows that the difference in demonstrated spores between the control suspension and the emulsified suspension disappears when the shaking is performed in the above manner. The presence or absence of tween 80 in the culture medium makes no difference. The first horizontal row shows that the surmised influence of tween 80 on the multiplication of spores of *B. subtilis* was only a chance finding. The fact that the difference found in table I amounted to merely one tube infected or not had already pointed in this direction.





## DISCUSSION.

For the demonstrating of an infection in an emulsion of the water/oil type it is necessary to shake this emulsion with a large amount of culture medium. The presence of an emulsifier of the tween type is not necessary, so the oil does not need to remain finely divided in the water; a short-lasting fine dispersion of the oil in the water gives the spores of *B. subtilis* sufficient opportunity to enter the water-phase. The tests described here have not given up till now any certainty that anaerobic organisms can be detected in this way. The strong aeration during the shaking procedure might hamper the anaerobic organisms in their multiplication. This is not a major objection. It is essential that both phases be sterile before making the water/oil emulsion. The oil phase can be adequately sterilised by heating and the water phase can be controlled for sterility in the normal way. The final control serves only to eliminate possible faults in the sterile handling during the making of the emulsion or the filling into final containers. The probability that only anaerobic organisms would contaminate the emulsion during these procedures is very slight. If it is absolutely necessary to demonstrate anaerobic organisms in the final emulsion, one can resort to incubating in an anaerobic jar.

The technical assistance of Miss N. VAN MINNEN is gratefully acknowledged.

## S u m m a r y.

To give the desired sensitivity to the sterility control of water/oil emulsions for injection purposes, it is absolutely necessary to shake the emulsion in the sterility medium. With the aid of a spore suspension of *B. subtilis* this has been demonstrated on a quantitative basis.

## L i t e r a t u r e.

1. ERNST, O. 1943. Zentralbl. f. Bakt. I. **150**, 155.
2. QUARLES VAN UFFORD, W. J. 1946. Ned. Tijdschr. v. Geneesk. **90**, 433.
3. SALK, J., LAURENT, A. M. and BAILEY, M. L. 1951. Am. J. Publ. Hlth. **41**, 669.
4. Standard Methods of Water Analysis. 1936. Am. Publ. Hlth. Ass. p. 205.

(Laboratoire de Microbiologie, Université de Louvain, Héverlé-lez-Louvain, Belgique).

## ÉTUDE DU MÉTABOLISME D'ACIDES AMINÉS CHEZ *ASPERGILLUS ORYZAE*

### III. ACIDES AMINÉS LIBRES DANS LE MYCÉLIUM CULTIVÉ SUR DIVERSES SOURCES DE CARBONE EN PRÉSENCE D'AMMONIAQUE<sup>1)</sup>

par

PAUL SIMONART et KWANG YŪ CHOW

(Reçu le 23 Juin 1953).

Il a été montré (2) que le mycélium d'*Aspergillus oryzae* cultivé sur tel ou tel acide aminé contient à l'état libre de l'acide glutamique, de l'alanine et souvent de l'acide aspartique ainsi que de la glutamine à côté d'autres acides aminés. Ces résultats soulignent le rôle joué par les réactions de transamination dans le métabolisme de cette moisissure, et par conséquent aussi l'interdépendance des métabolismes ternaire et quaternaire. C'est pourquoi il paraissait intéressant d'étudier la formation d'acides aminés par *Asp. oryzae* aux dépens d'ammoniaque et cela en présence de sources de carbone différentes, notamment en présence d'acide acétique, d'acide pyruvique, d'acide succinique, d'acide fumarique, d'acide  $\alpha$ -cétoglutarique et de glucose. Ce sont ces expériences qui font l'objet de la présente note.

Les méthodes expérimentales appliquées dans cette étude sont les mêmes que celles qui furent utilisées dans les recherches antérieures (1, 2). On laisse se développer *Asp. oryzae* sur flux continu d'une solution à 3% de bacto-casamino acids (Difco), puis on cultive la moisissure sur un liquide de substitution contenant ou bien le sel ammoniacal d'un acide organique à la concentration 0,5 M, ou bien 5% de glucose et 0,4% de sulfate d'ammoniaque.

Les résultats obtenus sont résumés ici tandis que les figures 1 à 12 reproduisent les chromatogrammes correspondants.

Lorsque la solution de substitution contient de l'acétate ammoniacal le mycélium renferme (fig. 1) après 24 heures de cul-

1) Part I, II: Antonie van Leeuwenhoek **19**, 121, 245, 1953.



Chromatogrammes d'acides aminés libres dans le mycélium d'*Aspergillus oryzae*, formé sur 3% de bacto-casamino acids à pH = 6,3 et cultivé ensuite de un à trois jours, sur flux continu d'une solution ammoniacale.

Fig. 1: Culture de un jour sur flux continu d'une solution 0,5 M d'acétate d'ammonium;

Fig. 2: Culture de trois jours sur flux continu d'une solution 0,5 M d'acétate d'ammonium;

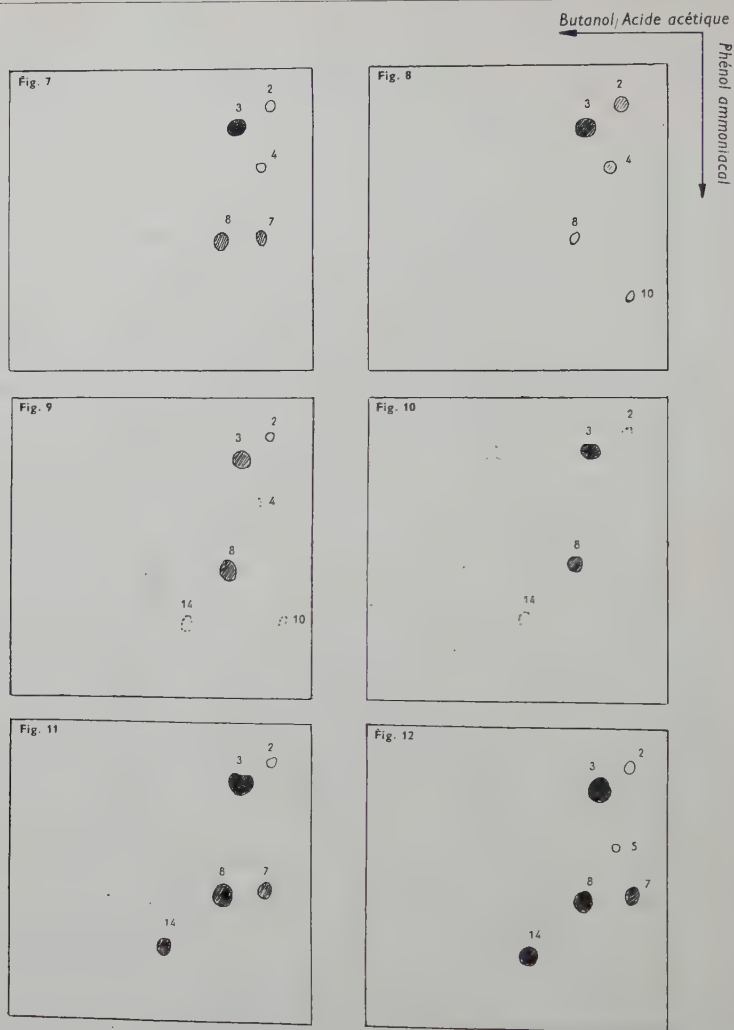
Fig. 3: Culture de un jour sur flux continu d'une solution 0,5 M de pyruvate d'ammonium;

Fig. 4: Culture de trois jours sur flux continu d'une solution 0,5 M de pyruvate d'ammonium;

Fig. 5: Culture de un jour sur flux continu d'une solution 0,5 M de succinate d'ammonium;

Fig. 6: Culture de trois jours sur flux continu d'une solution 0,5 M de succinate d'ammonium;

1 = cystéine; 2 = acide aspartique; 3 = acide glutamique; 4 = sérine; 5 = glycine; 7 = glutamine; 8 = alanine; 10 = ornithine; 11 = lysine; 12 = arginine; 14 = acide  $\gamma$ -aminobutyrique; 21 = spot non identifié; 22 = spot non identifié;



Chromatogrammes d'acides aminés libres dans le mycélium d'*Aspergillus oryzae*, formé sur 3% de bacto-casamino acids à pH = 6,3 et cultivé ensuite de un à trois jours, sur flux continu d'une solution ammoniacale.

Fig. 7: Culture de un jour sur flux continu d'une solution 0,5 M de fumarate d'ammonium;

Fig. 8: Culture de trois jours sur flux continu d'une solution 0,5 M de fumarate d'ammonium;

Fig. 9: Culture de un jour sur flux continu d'une solution 0,5 M de  $\alpha$ -céto-glutarate d'ammonium;

Fig. 10: Culture de trois jours sur flux continu d'une solution 0,5 M de  $\alpha$ -céto-glutarate d'ammonium;

Fig. 11: Culture de un jour sur flux continu d'une solution à 5% de glucose contenant 0,4% de sulfate d'ammonium;

Fig. 12: Culture de trois jours sur flux continu d'une solution à 5% de glucose contenant 0,4% de sulfate d'ammonium;

2 = acide aspartique; 3 = acide glutamique; 4 = sérine; 5 = glycine; 7 = glutamine; 8 = alanine; 10 = ornithine; 14 = acide  $\gamma$ -aminobutyrique.



ture, l'acide aspartique, l'acide glutamique, l'alanine et la glutamine à côté de traces de glycine, d'acide  $\gamma$ -aminobutyrique et d'ornithine. Après trois jours de culture (fig. 2) on retrouve, mais apparemment en quantité plus grande, les mêmes acides aminés qu'après 24 heures de culture, et en plus de cela se décèlent encore la sérine et la cystine ou cystéine.

Utilisant une solution de *p y r u v a t e a m m o n i q u e* comme liquide de substitution, on décèle dans le mycélium après 24 heures de culture (fig. 3) de la lysine en plus des acides aminés que l'on obtient pour l'acétate ammonique, sauf cependant l'acide  $\gamma$ -aminobutyrique. Après trois jours de culture les chromatogrammes montrent (fig. 4) la présence d'acide aspartique, d'acide glutamique, d'alanine, de glutamine, de glycine en quantités importantes, ainsi que de l'acide  $\gamma$ -aminobutyrique, de la cystine ou de la cystéine, de l'ornithine et des traces de lysine.

Avec le *s u c c i n a t e a m m o n i q u e*, on trouve dans le mycélium après 24 heures de culture (fig. 5), l'acide aspartique, l'acide glutamique, l'alanine, la glutamine, la lysine, l'acide  $\gamma$ -aminobutyrique et des traces de glycine et de cystine ou de cystéine; en outre, il y a présence d'une substance non identifiée, non hydrolysable dont le Rf dans le phénol est voisin de 1. Après trois jours de culture on ne retrouve (fig. 6) plus ce corps pas plus que l'acide  $\gamma$ -aminobutyrique et la cystine, mais en plus des acides aminés décelés après 24 heures de culture, on y met en évidence la sérine, l'ornithine, l'arginine et une substance non identifiée dont le spot est voisin de celui de l'acide glutamique.

Le *f u m a r a t e a m m o n i q u e* donne lieu après 24 heures de culture (fig. 7) à la présence d'acide aspartique, d'acide glutamique, d'alanine, de glutamine et de sérine. Après trois jours de culture (fig. 8) on ne retrouve plus de glutamine, mais, par contre, on décèle de l'ornithine; en outre, il semble qu'il y ait moins d'alanine mais plus d'acide aspartique et de sérine que dans la culture de 24 heures.

Lorsque le liquide de substitution contient l' $\alpha$ -*c é t o g l u t a r a t e a m m o n i q u e*, on décèle dans le mycélium après 24 heures de culture (fig. 9) l'acide aspartique, l'acide glutamique, l'alanine et des traces d'acide  $\gamma$ -aminobutyrique, d'ornithine et de sérine; après trois jours de culture (fig. 10) on ne trouve que l'acide glutamique et l'alanine avec des traces d'acide aspartique et d'acide  $\gamma$ -aminobutyrique.

Quand la solution de substitution contient 5% de glucose et 0,4% de sulfate d'ammonium on trouve après 24 heures de culture (fig. 11) de l'acide glutamique, l'alanine, l'acide aspartique, la glutamine et l'acide  $\gamma$ -aminobutyrique; après trois jours on trouve de la glycine en plus des mêmes substances, qui paraissent plus abondantes qu'après 24 heures.

De ces divers résultats il convient de faire ressortir les points suivants:

1. *Asp. oryzae* synthétise rapidement plusieurs acides aminés aux dépens d'ammoniaque et d'acides organiques ou de glucose.

2. Sauf pour l' $\alpha$ -cétoglutarate ammonique, on trouve dans le mycélium plus d'acides aminés différents lorsque l'on emploie le sel ammoniacal d'un acide ternaire plutôt qu'un acide aminé correspondant.

L'acétate, le pyruvate, le succinate et le fumarate ammoniques donnent lieu respectivement à l'apparition de plus nombreux acides aminés que la glycine, l'alanine et l'acide aspartique (2).

3. Dans le cas du pyruvate ammonique on ne trouve dans le mycélium après 24 heures de culture que peu d'alanine, mais cet acide aminé s'y accumule abondamment en trois jours. Ceci semblerait indiquer que l'alanine prend naissance par la réaction de transamination suivante qui fait encore ressortir un aspect du rôle multiple joué par l'acide glutamique:

$$\text{Acide glutamique} + \text{acide pyruvique} \rightarrow \text{acide } \alpha\text{-cétoglutarique} + \text{alanine}$$

De nombreux essais pour déceler par chromatographie l'acide  $\alpha$ -cétoglutarique dans le mycélium n'ont donné de résultats positifs que lorsque la moisissure se cultive sur une solution d'acide pyruvique; avec toutes les autres sources de carbone, il n'a pu être mis en évidence.

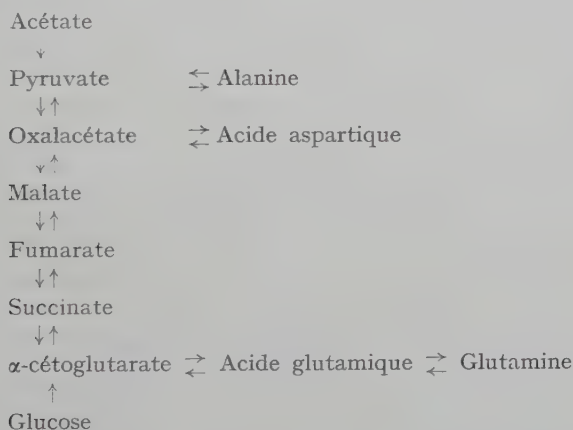
Pour rechercher l'acide  $\alpha$ -cétoglutarique, on chromatographie en prenant comme solvant un mélange de deux parties de butanol et d'une partie d'acide formique, et on révèle par vaporisation d'une solution à 0,5% de 2,4-dinitrophénylhydrazine dans HCl 2N. L'acide  $\alpha$ -cétoglutarique donne dans ces conditions un spot jaune de  $R_f = 0,68$ .

4. L'acide succinique donne lieu à la formation de plus d'acides aminés différents que l'acide fumarique, et celui-ci à plus que l'acide  $\alpha$ -cétoglutarique. Ce dernier acide constitue cependant une

bonne source de carbone, car après trois jours de culture il n'était plus décelable dans le liquide de substitution.

5. On retrouve pour toutes les substances essayées, l'acide glutamique, l'acide aspartique, l'alanine et la glutamine. Dans les grandes lignes, le métabolisme des diverses substances étudiées est assez analogue, ce qui revient à dire que l'acide acétique, l'acide pyruvique, l'acide succinique, l'acide fumarique, l'acide  $\alpha$ -cétooglutarique et le glucose se remplacent l'un l'autre pour la synthèse des acides aminés. Et il est à signaler ici que lors d'essais faits avec une solution de butyrate ammonique comme liquide de substitution, on n'a pu déceler dans le mycélium aucun acide aminé libre si ce n'est une trace d'acide glutamique.

L'équivalence comme source de carbone des diverses substances étudiées, d'une part, et la présence constante dans le mycélium d'alanine, d'acide aspartique et d'acide glutamique d'autre part, suggère le schéma de métabolisme suivant qui montre l'interdépendance de substances ternaires entre elles et leur rapport avec les acides aminés:



Il est cependant à remarquer que l'acide oxalacétique et l'acide malique n'ont pas été étudiés dans cette recherche-ci, et que par conséquent les réactions qu'ils supposent, reposent sur des considérations théoriques. Mais il convient néanmoins d'ajouter que dans les mycélium analysés on a pu mettre en évidence par chromatographie un acide dont le  $R_f$  est identique à celui de l'acide malique, et qui s'identifie probablement avec lui.

Ce schéma rattache le métabolisme ternaire au métabolisme quaternaire par les réactions de transamination. Celles-ci jouent, en effet, probablement un rôle primordial dans le métabolisme étudié, ainsi que cela ressortait déjà de l'étude de divers acides aminés en tant que source d'azote et de carbone (2).

### R é s u m é.

*Aspergillus oryzae* transforme rapidement l'ammoniaque en groupement aminé.

Lorsqu'une colonie préformée de cette moisissure se cultive sur une solution du sel ammoniacal d'un des acides suivants: acides acétique, pyruvique, succinique, fumarique,  $\alpha$ -cétoglutarique ou encore sur glucose en présence de sulfate ammonique, on trouve dans le mycélium plusieurs acides aminés à l'état libre. Dans ces conditions, on y décèle toujours l'alanine, l'acide aspartique, l'acide glutamique et la glutamine; en outre, on y met éventuellement en évidence d'autres acides aminés tels glycine, sérine, arginine, acide  $\gamma$ -aminobutyrique.

L'acétate, le pyruvate, le succinate et le fumarate ammoniques, donnent lieu respectivement à l'apparition de plus nombreux acides aminés libres dans le mycélium que la glycine, l'alanine et l'acide aspartique.

Les diverses substances essayées paraissent équivalentes en tant que sources de carbone, ce qui fait aussi ressortir le rôle des réactions de transamination dans le métabolisme étudié.

### B i b l i o g r a p h i e.

1. SIMONART, P. et CHOW, K. Y. 1953. Ant. van Leeuwenhoek **19**, 121.
  2. SIMONART, P. et CHOW, K. Y. 1953. Ant. van Leeuwenhoek **19**, 245.
-

(Department of Bacteriology and Experimental Pathology, Netherlands' Institute for Preventive Medicine, Leiden).

## EXPERIMENTAL INFECTION OF SUCKLING-FERRETS WITH VIRUSES OF THE COXSACKIE GROUP ISOLATED FROM CASES OF INFANTILE GASTROENTERITIS

by

**J. D. VERLINDE and LIE KHING TING**

(Received October 5, 1953).

The etiology of epidemic infantile gastroenteritis seems to be subject to considerable variation. Although *E. coli* type Bray is considered to play an important part, many investigators hesitate to attribute either a primary, or a unique etiological significance to this micro-organism (1, 5). Occasionally, certain viruses have been claimed as possible etiologic agents. BUDDINGH and DODD (2) recovered a virus producing keratitis in rabbits following corneal inoculation. LIGHT and HODES (4) reported the isolation of a filterable agent, which was transmissible to calves. VERLINDE (6) succeeded in producing illness and death in suckling-ferrets following oral infection with stool filtrates, while convalescent serum from children protected these animals against lethal infection. Finally, HODES, ZEPP and FLORMAN (3) isolated a virus producing encephalitis in 17-day old mice. None of the viruses has been identified, and none of the findings has been confirmed or denied.

In 1951, several strains of group A Coxsackie virus, mostly of type 2, have been isolated in this laboratory from the stools of children suffering from illnesses, which were frequently associated with diarrhea, and which had frequently been diagnosed as either summer grippie or Bornholm's disease (7).

One year later, when examining stools from children with diarrhea in a children's clinic, *E. coli* type Bray was isolated from the stools of 21% of 114 children with diarrhea. Six pools were made from the stools of 36 children with diarrhea, from which we failed to isolate



*E. coli* type Bray. From one of the pools, however, a group A Cocksackie virus was recovered by inoculation of suckling-mice.

These observations returned our thoughts to the cases of infantile diarrhea, from which we isolated in 1948 a virus pathogenic for suckling-ferrets (6). Since no data were available on the susceptibility of suckling-ferrets for *E. coli* type Bray and for Cocksackie viruses, we carried out a limited number of infection experiments with these agents in newborn ferrets, dogs and cynomolgus monkeys.

We failed to produce any signs of illness in newborn ferrets and dogs by oral administration of freshly isolated cultures of *E. coli* type Bray. Moreover, newborn dogs and 3 newborn cynomolgus monkeys proved insusceptible for oral and intramuscular inoculation with strains of group A type 2, and group B type 1 Cocksackie virus. These strains, however, proved pathogenic for 2-day old suckling-ferrets when administered either orally, or subcutaneously.

Four groups of 3 suckling-ferrets were infected either by oral, or by intramuscular route with either group A type 2, or group B type 1 Cocksackie virus. The inoculum consisted of a 10% suspension of suckling-mouse carcass, the titre being  $10^{-5}$  to  $10^{-6}$  for suckling-mice. The amount inoculated in suckling-ferrets was 0.1 ml by the intramuscular route, and 0.5 ml by the oral route.

#### I. Group A type 2 virus subcutaneously.

The animals showed weakness of the limbs on the 10th day following inoculation. They were apparently unable or unwilling to suck. One animal died and the others were sacrificed on the 10th day. Pooled carcass suspension in a dilution of  $10^{-1}$  was inoculated into 7 suckling-mice, of which only one showed clinical signs of Cocksackie A virus infection. At histological examination, the characteristic muscle lesions of group A Cocksackie virus infection were found in the mice, and their carcasses produced a rapidly fatal group A Cocksackie virus infection in other suckling-mice.

Histologic examination of the suckling-ferrets showed edema, shrivelling, loss of transverse striation and eosinophilic degeneration of muscle fibres. The muscle lesions, however, were less generalized and less extensive than those produced by the same strain in suckling-mice.

#### II. Group A type 2 virus per os.

Clinical symptoms and histologically demonstrable lesions similar to those in the first experiment developed on the 10th and 12th day following inoculation. No virus could be recovered from the carcasses.

### III. Group B type 1 virus subcutaneously.

Dullness developed on the 10th day; one animal was found dead, and the others were sacrificed on the same day. No virus could be recovered from the carcasses and the brains.

At histologic examination no evidence of muscular involvement was found. The pancreas, however, showed serious degeneration with beginning fibrosis, and circumscript areas with lysis of ganglion cells were found in the cerebral cortex.

### IV. Group B type 1 virus per os.

Dullness appeared on the 10th day. The animals died 2 days later. Virologic examination was negative. The brains were heavily involved; almost all ganglion cells had partly or completely disappeared in fairly large, sharply outlined areas of the cerebral cortex. No muscular and pancreatic involvement was found.

## Discussion.

Suckling-ferrets proved susceptible for both group A and group B strains of Cocksackie virus, which caused illness and death. Yet, the viruses do not seem to maintain themselves as suckling-mouse pathogenic agents, since only the group A type strain could be recovered from suckling-ferrets with difficulty, and the titre for suckling-mice had dropped from  $10^{-6}$  to  $10^{-1}$ . Since no more suckling-ferrets were available, serial passages in these animals could not be made.

The observation, that Cocksackie viruses may produce a fatal infection in suckling-ferrets suggests, that the agent isolated by one of us from the stools of cases of epidemic infantile gastroenteritis in 1948, by inoculation of suckling-ferrets, has been a strain of this group of viruses.

The, perhaps, occasional presence of Cocksackie virus in stools from cases of infantile gastroenteritis, is in itself no proof that it is one of the etiologial agents of this disease. More information as to the possible etiologial significance of Coxackie virus in infantile gastroenteritis is necessary.

## Summary.

Group A Cocksackie viruses have been isolated from the stools of patients suffering from infantile gastroenteritis. Strains of both group A and group B Cocksackie virus produced illness and death in

suckling-ferrets, but the pathogenicity of the virus for suckling-mice falls rapidly in the ferret. Group A virus produced in suckling-ferrets slight muscle lesions, and group B virus produced pancreatic necrosis and lysis of nerve cells of the cerebral cortex.

### References.

- BEEUWKES, H., GIJSBERTI HODENPIJL, A. K. A. en TEN SELDAM, R. E. J. 1949. Maandschr. Kindergeneesk. **17**, 195.
- BUDDINGH G. J. and DODD, K. 1944. Journ. Pediatr. **25**, 105.
- HODES, H. L., ZEPP, H. D. and FLORMAN, A. L. 1952. Amer. Journ. Dis. Childr. **83**, 65.
- LIGHT, J. S. and HODES, H. L. 1943. Amer. Journ. Publ. Health **33**, 1451.
- STOPPELMAN, M. R. H. and VAN DER PLAATS, A. B. J. 1953. Acta Paediatr. **42**, 215.
- VERLINDE, J. D. 1948. Maandschr. Kindergeneesk. **16**, 385.
- VERLINDE, J. D. and VAN TONGEREN, H. A. E. 1952. Antonie van Leeuwenhoek **18**, 239.
-

(Department of Bacteriology and Public Health, Washington State  
College, Pullman, Washington).

## EXPERIMENTS ON THE MICROBIOLOGY OF CELLULOSE DECOMPOSITION IN A MUNICIPAL SEWAGE PLANT <sup>1)</sup>

by

**LEROY R. MAKI <sup>2)</sup>**

(Received September 1, 1953).

Municipal sewage plants often function improperly when wastes from domestic sources are augmented by those from the food industries (6). The disturbance may be due to overloading of the plant but it is also possible that the nature of the materials in the industrial waste is an important factor. As a first step toward understanding the effects of adding food processing wastes to sewage, it has seemed essential to study the microbial and biochemical processes in municipal plants receiving chiefly domestic waste. The anaerobic breakdown of the solids, particularly cellulose, has been the primary concern of the present investigation.

In order to determine which constituents of sewage are quantitatively important substrates for bacterial action, chemical analyses for hemicellulose (fraction soluble in 5 %  $\text{H}_2\text{SO}_4$ ), cellulose (soluble in 70 %  $\text{H}_2\text{SO}_4$ ), protein (calculated as 6 times the total N), lipids (ether extractible fraction), and ash, were performed on two representative samples of the sewage solids obtained from the Pullman municipal sewage disposal plant. The results are shown in table 1.

The high total recovery of material shown in tabel 1 is due to overlapping between the various fractions. It is significant that cellulose comprises almost half of the organic material entering the digestor. A study of the cellulose fermentation appears essential to

---

<sup>1)</sup> This investigation was supported in part by a research grant from the National Institute of Health, U.S. Public Health Service.

<sup>2)</sup> Present address: Department of Bacteriology, University of Wisconsin, Madison, Wisconsin.

TABLE 1.  
The Results of Analyses of Sewage Solids.

|               | Sample I | Sample II | Average |
|---------------|----------|-----------|---------|
| Hemicellulose | 5.60%    | 6.70%     | 6.15%   |
| Cellulose     | 36.18    | 32.78     | 34.48   |
| Lipids        | 14.01    | —         | 14.01   |
| Protein       | 19.02    | 18.95     | 18.98   |
| Ash           | 34.80    | 34.95     | 34.88   |
| Total         |          |           | 108.50  |

an understanding of the normal biochemical and biological processes of sewage solids decomposition.

#### Methods for culture counts and isolation.

Before 1944 isolations of anaerobic cellulolytic organisms were occasionally accomplished by indirect methods. Recently, classic techniques have been adapted to their culture and they can be easily isolated (5). A culture count in agar dilution series can be obtained without prior enrichment. HUNGATE (5) isolated three strains of cellulolytic bacteria from sludge taken from a secondary anaerobic digester. The fermentation products were determined for two of the strains but the nutritional requirements and carbohydrates fermented were not studied. The results of a more extended investigation of the cellulolytic bacteria in sludge, with experiments on possible factors influencing their activity in the normal fermentation, are the subject of the present report.

Several different cellulose media have been tested in an attempt to demonstrate the maximum numbers of cellulolytic bacteria in sludge. These have been prepared and inoculated according to the anaerobic culture methods previously described (5). Best results were obtained when sludge was serially diluted into a medium prepared as follows: 40 ml tapwater containing 0.4 g finely divided cellulose (Whatman's No. 1 filter paper ground wet in a pebble mill) were added to 30 ml of supernatant liquid obtained by letting the solids settle out of the effluent from the primary digester of the Pullman sewage plant. Thirty ml of a solution of inorganic salts were added so that the final concentrations were 0.1 % NaCl, 0.1 %  $(\text{NH}_4)_2\text{SO}_4$ , 0.05 %  $\text{K}_2\text{HPO}_4$ , 0.01 %  $\text{MgSO}_4$ , and 0.01 %  $\text{CaCl}_2$ .



Yeast extract 0.2 %, agar 1.5 %, and resazurin 0.0001 %, were the other ingredients of the medium. Sterile sodium carbonate (0.25 % final concentration) was added after sterilization, and a reducing agent was introduced just after inoculation. Cysteine, sodium sulfide, and sodium formaldehyde sulfoxylate have been used with equal effectiveness as reducing agents. The inoculum was obtained from the sewage plant and cultured within a few hours of collection. Cultures were incubated at 38 C. Cellulose-decomposing colonies were detected by the clearing of the surrounding cellulose.

#### Numbers of cellulolytic bacteria in sludge.

Using the above methods counts have been made on sewage at various stages of treatment. The counts for three series inoculated with material from the primary digester of the Pullman plant were 140,000, 16,000, and 168,000 cellulolytic bacteria per ml, respectively. The inoculum for each of these cultures was obtained separately, with an intervening period of several days between each sampling. Another sample taken a few months later from the same digester gave a culture count of 970,000.

Sludge from the bottom of the secondary digester gave a culture count of 4,000 cellulolytic colonies per ml. A count on the raw sewage entering the Pullman plant disclosed no cellulolytic organisms, using an inoculum of 0.1 ml. Culture series inoculated from the primary tank of the Moscow, Idaho, sewage plant showed about 100,000 cellulose digesters per ml.

Due to the tendency of the cellulose bacteria to adhere to the fibers, the culture count for these organisms in the sludge is probably much lower than the number of cells present.

#### Nature of the cellulolytic bacteria.

The cellulolytic bacteria seen in cultures inoculated with sludge from the Moscow and Pullman sewage plants were similar in morphology and resembled the strains previously described (5). Ten cellulolytic strains were isolated in pure culture from sludge obtained from the Pullman plant at different times. These strains represented the cellulolytic flora over rather a long period.

These strains showed colonies in cellulose agar after four days of incubation. Seven or eight days were required for the production of a colony of sufficient diameter to make it easy to subculture. The isolated organisms may be separated into two groups according to

their colony characteristics. One type, after two weeks of incubation, digests enough cellulose to produce a clear zone of considerable diameter around the colony. These active strains will completely digest all of the cellulose in four to six weeks. Strains of the other type are less actively cellulolytic. They give only a narrow zone of dissolved cellulose after a month of incubation.

Oval spores were observed in active strain 4*b* when it was first isolated. Subsequently, the number of spores diminished to the point where they could no longer be detected microscopically. However,

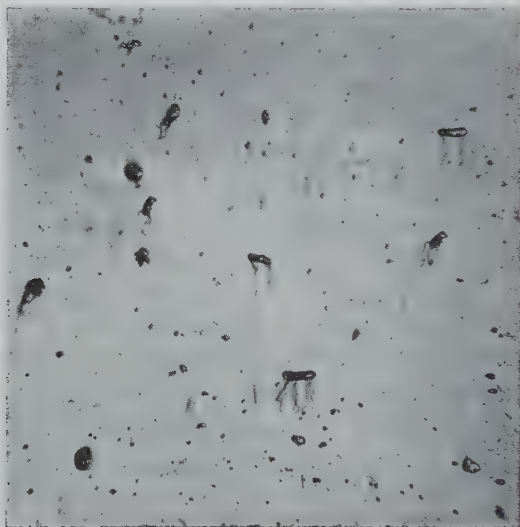


Fig. 1. Flagella stain of an actively cellulolytic strain from sludge.

inocula for subcultures were viable after pasteurization, indicating the presence of at least a few spores. This strain remained viable for several months without transfer whereas the others were often inviable if not transferred at intervals of less than a month.

The less actively cellulolytic strains were non-motile whereas motility was easily detected in the others when examined under anaerobic conditions. Flagellation was peritrichous (fig. 1).

A summary of the characteristics found for three actively cellulolytic strains and two of the less active strains is given in table 2.

The three active strains were grown in cultures suitable for quantitative analyses and the fermentation products were de-

TABLE 2.

| Strain                            | High cellulolytic activity |       |          | Low cellulolytic activity |       |
|-----------------------------------|----------------------------|-------|----------|---------------------------|-------|
|                                   | 4b                         | 13a   | E        | 13s                       | 13c   |
| Gram reaction                     | —                          | —     | —        | —                         | —     |
| Length in microns                 | 3—12                       | 3—12  | 3—12     | 3—12                      | 3—12  |
| Diameter in microns               | 0.8—2                      | 0.8—2 | 0.8—2    | 0.8—2                     | 0.8—2 |
| Oval spores in microns            | 0.8 × 1.0                  | —     | —        | —                         | —     |
| Motility <sup>1)</sup>            | +                          | +     | +        | —                         | —     |
| Action on litmus milk             | None                       | None  |          |                           |       |
| Production of indole              | None                       | None  |          |                           |       |
| Nitrites from nitrates            | None                       | None  |          |                           |       |
| Action on nutrient gelatin        | None                       | None  |          |                           |       |
| Growth on nutrient agar           | None                       | None  |          |                           |       |
| Growth in nutrient broth          | None                       | None  |          |                           |       |
| Optimum pH                        |                            |       | 6.8      |                           |       |
| pH range                          |                            |       | 6.2—7.8  |                           |       |
| Optimum temperature               |                            |       | 30—37°C. |                           |       |
| Temperature range                 |                            |       | 23—40°C. |                           |       |
| Inulin fermentation <sup>2)</sup> | —                          | +     | —        | —                         | —     |
| Esculin fermentation              | —                          | +     | —        | —                         | —     |

<sup>1)</sup> Motility when present is by peritrichous flagella. See fig. 1.

<sup>2)</sup> All strains fermented cellulose, cellobiose, starch, maltose, and dextrin. None of the strains fermented glucose, trehalose, l-arabinose, d-xylose, fructose, galactose, mannose, rhamnose, inositol, salicin, sucrose, lactose, raffinose, glycerol, d-sorbitol, dulcitol, or mannitol.

TABLE 3.

Results of analyses for cellulose fermentation products in mmol of three strains.

|                        | Strain E |       | Strain 4b |       | Strain 13a |       |
|------------------------|----------|-------|-----------|-------|------------|-------|
|                        | Control  | Exp.  | Control   | Exp.  | Control    | Exp.  |
| H <sub>2</sub>         | 0.015    | 0.261 | 0.045     | 0.114 | 0.028      | 0.229 |
| CO <sub>2</sub>        | 0.238    | 0.865 | 0.186     | 0.493 | 0.234      | 0.969 |
| Ethanol <sup>1)</sup>  |          | 0.663 | 0.176     | 0.325 | 0.059      | 0.598 |
| Formic Acid            | 0.224    | 0.075 | 0.192     | 0.041 | 0.138      | 0.565 |
| Acetic Acid            | 0.287    | 0.442 | 0.263     | 0.766 | 0.354      | 0.395 |
| Lactic Acid            | 0.036    | 0.098 | 0.033     | 0.038 | 0.083      | 0.111 |
| Cellulose disappearing | 58.9 mg  |       | 64.6 mg   |       | 89.9 mg    |       |
| C recovery             | 67.8 %   |       | 68.4 %    |       | 72.4 %     |       |

<sup>1)</sup> sample lost.

TABLE 4.

Comparison of cellulose fermentation products in mmol formed by the three strains.

| Strain          | E                   | 4b     | 13a   |
|-----------------|---------------------|--------|-------|
| H <sub>2</sub>  | 0.246               | 0.069  | 0.201 |
| CO <sub>2</sub> | 0.627               | 0.307  | 0.735 |
| Ethanol         | 0.500 <sup>1)</sup> | 0.149  | 0.539 |
| Formic Acid     | -0.149              | -0.151 | 0.427 |
| Acetic Acid     | 0.135               | 0.503  | 0.041 |
| Lactic Acid     | 0.062               | 0.005  | 0.028 |

<sup>1)</sup> estimated.

terminated for each, using the methods previously described (5). The results of the analyses are given in table 3 and the fermentation products of the three strains are compared in table 4.

The principal products of cellulose fermentation by the three strains are hydrogen, carbon dioxide, ethanol, and acetic acid. The relatively high values for formic acid in the control cultures are probably due to fermentation of the yeast extract, phytone, and trypticase (see p. 191) which were constituents of the medium. Strains E and 4b produced less formic acid in the presence of cellulose whereas 13a produced more. Strain 4b formed relatively less hydrogen, CO<sub>2</sub>, ethanol and lactic acid, but more acetic acid.

#### Nutritional requirements: Effect of salts.

The influence of the concentration of inorganic salts on growth and cellulose digestion by strain E was tested. Two experiments were set up on a Latin Square design. In the first the concentrations of sodium chloride and ammonium sulfate were varied while maintaining fixed amounts of calcium chloride and magnesium sulfate. The basal medium was a broth containing cellulose, yeast extract, phytone and trypticase in 0.2 % concentrations; calcium chloride and magnesium sulfate in 0.01 % concentrations; and KH<sub>2</sub>PO<sub>4</sub> in 0.05 % concentration, respectively. The percentages of sodium chloride and ammonium sulfate used and the days for complete fermentation of the cellulose are shown in table 5.

Although the results in table 5 show some unexplained variations they suggest that a concentration of 0.04 % is optimal for both ammonium sulfate and sodium chloride.

TABLE 5.

Time in days required for cellulose fermentation at various salt concentrations.

| % Ammonium sulfate | % Sodium Chloride |      |      |      |      |      |
|--------------------|-------------------|------|------|------|------|------|
|                    | 0.01              | 0.02 | 0.04 | 0.06 | 0.10 | 0.20 |
| 0.01               | 6                 | 6    | 5    | 6    | 6    | 10   |
| 0.02               | 6                 | 6    | 6    | 6    | 6    | 6    |
| 0.04               | 6                 | 5    | 5    | 6    | 6    | 6    |
| 0.06               | 6                 | 6    | 5    | 6    | 6    | 6    |
| 0.10               | 6                 | 6    | 6    | 6    | 10   | 6    |
| 0.20               | 10                | 6    | 6    | 10   | 10   | 6    |

In the second experiment the concentrations of sodium chloride and ammonium sulfate were maintained at 0.04 % and the concentrations of added magnesium sulfate and calcium chloride varied between 0 and 0.02 %. The higher concentrations of either of these salts gave a slightly increased rate of cellulose disappearance (6 days) as compared with no additions of either salt (8 days). Apparently the other ingredients of the culture medium contained sufficient amounts of these salts to give almost optimum growth.

#### Nutritional requirements: Effect of organic substances.

Preliminary attempts to grow the cellulolytic bacteria in liquid media failed, though the cultures could readily be grown in the same media with added agar. It was found that the addition of sterile undispersed agar to the liquid cultures would support the growth of strains of the more actively cellulose-digesting type. Phytone and trypticase <sup>1)</sup> were found capable of replacing the agar in liquid media inoculated with these strains. No combinations of nutrients were found which would give growth of the less active strains in liquid media.

Since all the bacterial strains isolated from sludge were presumably growing in it and nourished in part by dissolved material, it

<sup>1)</sup> Phytone and trypticase are produced by the Baltimore Biological Laboratories, phytone being a papaic digest of soya meal and trypticase a pancreatic digest of casein.



seemed possible that sludge liquor might support growth in liquid media. This was tested by separating the supernatant liquor after the sludge solids were allowed to settle, adding cellulose to it, sterilizing in the autoclave, and inoculating with the pure culture. No growth of any strains was obtained with the sludge liquor alone. With the addition of 0.2 % yeast extract good development of the less actively cellulolytic cultures was obtained if agar medium was used, but growth in liquid medium failed. Yeast extract agar alone was inadequate for all strains. These results indicate that the heat sterilized sludge supernatant liquor contained some growth-promoting materials, but required supplementation.

Although phytone and trypticase favored the growth of the pure cultures, they were found to exert an unfavorable effect on the development of mixed cultures containing the cellulolytic bacteria. When they were incorporated into the medium used for initial dilution series inoculated with sludge, fewer cellulolytic colonies developed. Presumably the rapid growth of the bacteria attacking these materials made the medium unfavorable for the cellulose digesters, perhaps by increasing the pH through liberation of ammonia from the nitrogenous constituents.

#### Nature of the products of cellulose digestion.

It has been reported (5,7) that there is an accumulation of reducing substances in old cellulose agar tubes after the fermentation of cellulose by a pure culture has ceased. The production of reducing sugars was tested by inoculating strain 13a into fifty ml of agar medium containing more cellulose than could be fermented. When fermentation ceased the cellulolytic enzymes continued to act and ultimately digested all of the cellulose. A water extract of the agar medium showed strong copper reduction.

The reducing material was identified on an ascension-paper chromatograph in parallel with glucose and cellobiose as controls. The developing solvent was composed of 40 ml n-butanol, 10 ml ethanol (95 %) and 50 ml of distilled water (9). After the column had developed for 18-20 hours at room temperature, the solvent line was marked, and the paper was dried in an oven at 100° C. Reducing sugars were located by spraying the paper with a mixture containing 0.5 g benzidine, 20 ml of glacial acetic acid, and 80 ml of absolute ethanol (4) and then drying at 100° C. for ten minutes to evaporate the reagents and develop the spots.

The Rf values for glucose and cellobiose were 0.36 and 0.17, respectively. The values for the sugar in the culture of 13a were found to be 0.38 in three separate determinations, indicating that glucose is a product of cellulose hydrolysis by this organism.

#### Rate of cellulose fermentation: Pure culture.

Since cellulose is one of the important constituents of sewage it seemed possible that its rate of decomposition might be a factor limiting the rate of sewage conversion. Three hundred ml of a medium containing yeast extract, trypticase, phytone, inorganic salts, and 0.2 % cellulose was placed in a 500 ml round bottom flask and autoclaved. Sodium carbonate was added to give a pH of 6.8 using carbon dioxide as the gas, and the medium was inoculated with an active culture of strain E. The flask was closed with a rubber stopper containing a self-sealing diaphragm cap. Sodium formaldehyde sulfoxylate was added with a sterile syringe and needle to reduce the medium. In withdrawing a sample, the cap was sterilized with 70 % ethanol and the aliquot for analysis was removed with a sterile syringe.

The cellulose in each aliquot was separated and weighed. The liquid was acidified, steam distilled, and the volatile acids titrated. Graphical representations of the results are shown in figures 2 and 3.

The culture showed an immediate increase in acid production which continued until all of the cellulose disappeared. The weight of unfermented cellulose decreased almost linearly with time until all of the cellulose disappeared. The decrease was 0.026 % cellulose per day. This is significantly

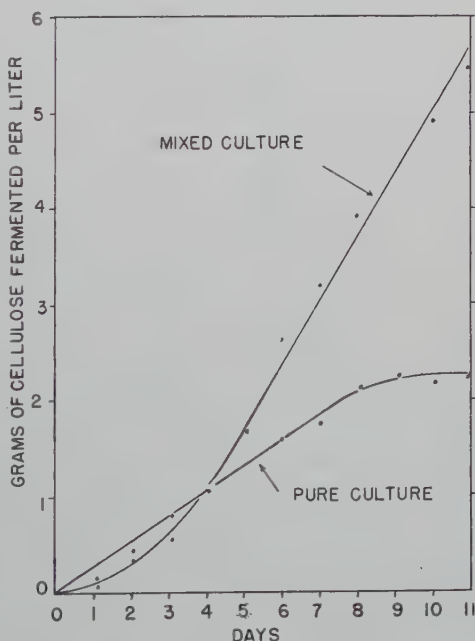


Fig. 2. The rate of cellulose fermentation by strain E in pure and mixed cultures.

less than the 0.1 % decrease found in sludge by BUSWELL (3). It seemed possible that the greater rate in sludge might be due to accompanying bacteria.

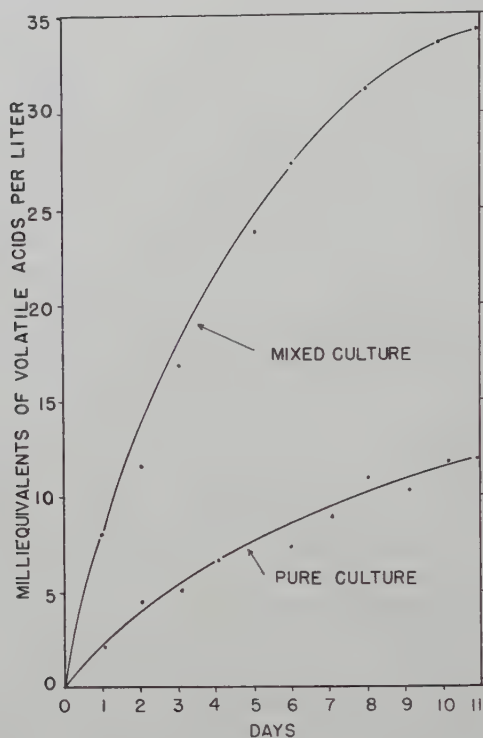


Fig. 3. The rate of acid production by strain E in pure and mixed cultures.

#### Rate of cellulose fermentation: Controlled mixed culture.

The influence of a non-cellulolytic bacterium from sludge on the rate of cellulose digestion by the cellulolytic strain was tested. A gram positive motile sporeformer which appeared in large numbers ( $5 \times 10^7$  per ml) was isolated from a dilution series inoculated with sludge from the primary tank. The organism and strain E were inoculated into a liquid culture containing the following percentages: cellulose, 1.1; phytone, 0.5; trypticase, 0.2; yeast extract, 0.2;  $\text{Na}_2\text{CO}_3$ , 0.25. Inorganic salts were the other ingredients of the medium. There was complete fermentation of the cellulose in twenty days. Previously strain E had never completely digested the cellulose in a liquid

medium when the concentration exceeded 0.25 %. This suggested that the sporeformer increased the rate of cellulose fermentation and a quantitative experiment was run to determine the magnitude of the effect.

Strain E and the clostridium were both inoculated into a medium containing 1 % cellulose and the amounts of cellulose and volatile acids in the culture were determined at intervals. The results of this experiment are also shown in figures 2 and 3.

The mixed culture gave a more rapid fermentation, with cellulose disappearing at a rate of 0.066 % per day. This is more than twice the rate for the pure culture and approaches the value reported by BUSWELL (3) for sludge. The results indicate that non-cellulolytic bacteria in sludge may increase the rate of cellulose fermentation.

The results of analyses of the mixed culture for fermentation products after three weeks of incubation are given in table 6. The gaseous products were not recovered.

TABLE 6.  
Analysis of the non-gaseous fermentation products of cellulose in 20 ml of the mixed culture of strain E and the Clostridium.

| Products     | Wt. mg | mmols |
|--------------|--------|-------|
| Ethanol      | 17.6   | 0.383 |
| Acetic Acid  | 31.5   | 0.526 |
| Butyric Acid | 27.7   | 0.315 |
| Formic Acid  | 6.2    | 0.134 |
| Lactic Acid  | 19.0   | 0.211 |

Comparison of the results in table 6 with those in table 3 shows that the presence of the clostridium caused the formation of a large amount of butyric acid. Since the butyric acid probably arose through condensation of two molecules of acetic acid, its occurrence as an end product diminishes the quantity of acetic acid produced from a given quantity of cellulose. The final concentration of acetic acid (plus acetate) was about 0.2 %. If accumulation of acetic acid tended to inhibit cellulose fermentation, its removal through conversion to butyric acid might explain the increased cellulose fermentation in the mixed culture. The effect of adding sodium acetate to cellulose cultures was tested.

### Effect of acetate on cellulose digestion.

Sodium acetate in varying amounts was added to a series of culture tubes containing 0.2 % trypticase, 0.5 % phytone, 0.2 % yeast extract, 0.1 % cellulose, 0.25 %  $\text{Na}_2\text{CO}_3$ , plus organic salts. These were inoculated with strain E and the time required for complete fermentation of the cellulose (0.2 %) was recorded. The results obtained have been plotted in figure 4.

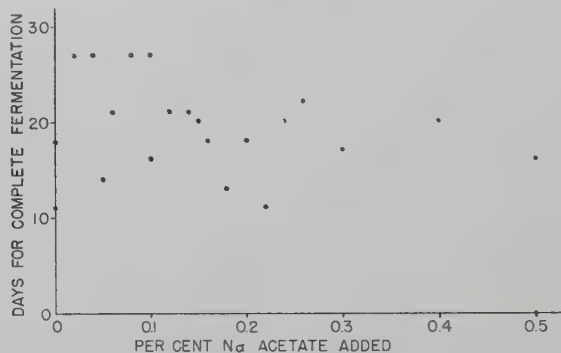


Fig. 4. The effect of sodium acetate on the rate of cellulose decomposition.

Inspection of the figure shows considerable variation and no trend which would suggest that the amounts of sodium acetate used were effective in inhibiting cellulose fermentation. Acetate ion is probably much less effective than the undissociated acid as an inhibitor of growth. The relatively narrow range of pH in which growth is initiated (table 1) and the fact that cultures do not cause a drop in pH below 5.5 suggest that an increase in acidity does limit growth and this is probably due to accumulation of undissociated acids. The experiment with sodium acetate shows that cellulose fermentation is not inhibited if the pH is maintained near neutrality.

It seems doubtful that removal of acetic acid by the clostridium is the chief cause of its favorable influence on the rate and total amount of cellulose fermentation since the concentration of acetic acid (0.2 %) reached in the mixed culture is much greater than in the pure cultures in which the total substrate dissimilated was never more than 0.25 %. Additional factors must be concerned, among which the elaboration of essential nutrients for the cellulose digesters may be important.



## DISCUSSION.

A similarity between the sporeforming and asporogenous strains of cellulose-fermenting organisms isolated from sewage is apparent, particularly in the pattern of substrates fermented. The non-spore-forming strains may be asporogenous varieties related to the spore-former. The fact that with the single sporogenous strain the number of spores produced rapidly diminished in laboratory culture suggests that in time an entirely asporogenous variety might evolve. The continuous supply of nutrients in the primary digester and the maintenance of favorable anaerobic conditions for growth could decrease the survival advantages of spore formation to such a point that a greater proportion of non-sporeforming variants would survive.

Whether isolated asporogenous strains have actually evolved from a sporeforming type cannot be decided with the information at hand. They are not uniformly alike; the less actively cellulolytic types being non-motile and more difficult to grow in liquid media, and the active types showing some variation in the ratios of fermentation products. A reliable taxonomic separation of the various types would require the isolation of more strains and completion of more extensive studies than were feasible during the present investigation.

The sewage entering the Pullman anaerobic digester averages 4 % solids <sup>1)</sup>. According to the analytical results shown in table 1 it would have a cellulose content of 1.4 % based on the wet weight. The growth curve studies with a mixed culture showed a rate of cellulose decomposition of 0.066 % per day. BUSWELL (3) set up a continuous feeding experiment with sludge in which cellulose was added at a rate of 0.105 % per day for a period of 86 days. He obtained a 95 % conversion of the cellulose to gases during this period. The greater rate of cellulose decomposition in sludge, as compared with that in the mixed culture of strain E and the clostridium, suggests that additional non-cellulolytic bacteria in sludge have a favorable influence on cellulose fermentation.

The volatile acids in BUSWELL's continuous cellulose fermentation experiment did not rise over 3000 parts per million in 86 days, whereas in the batch experiment with strain E and the clostridium, the volatile acids increased to this value in 12 days and then exceeded it. The further conversions of acids to methane by the mixed flora in

---

<sup>1)</sup> Based on information provided by the operator.

sludge probably account for at least part of the increased rate of cellulose fermentation in sludge.

According to the rate found by BUSWELL a 13-day cycle time would be needed to digest the cellulose entering the anaerobic digester of the Pullman plant. The cycle time in actual use is 41 days for the primary tank. This time plus the additional time the sewage remains in the secondary tank would appear to be more than ample for cellulose digestion. Whether other constituents of sewage require a longer cycle time than cellulose must await further investigation. The present results indicate that insofar as cellulose is concerned the cycle could be shortened.

The fermentation products of the cellulolytic bacteria from sludge are known to be attacked by methane bacteria. Hydrogen and carbon dioxide are metabolized by isolated pure cultures (2, 8, 10). Ethanol (1), formic acid (8, 10), and acetate (3, 8, 11) are readily dissimilated to methane by pure or mixed cultures and lactic acid is known to be decomposed to methane by mixed cultures (3). The rates at which formic acid and acetate are metabolized (8) indicate that the decomposition of these materials to methane occurs at a more rapid rate than does the fermentation of cellulose. Their rate of production from cellulose would not appear to exceed their rate of conversion to methane. This makes attractive the hypothesis that the initial breakdown of cellulose is a factor limiting the rate of conversion of sewage solids to methane and carbon dioxide. Studies on the other constituents of sewage, in addition to experimental determinations of minimum cycle time, will be necessary to test this hypothesis.

In anaerobic sludge there are undoubtedly many kinds of micro-organisms which carry out conversions not mentioned in the above paragraphs. Microscopic examination of a representative sample of sludge from the primary digester at Pullman revealed many long filamentous rods, numerous short motile rods, and a few cocci. Under darkfield, spirochetes of the genus *Leptospira* were observed in numbers exceeding 1000 per ml. On one occasion ciliate protozoa were seen. No spirilla were noted. A sample from the bottom of the second digester showed predominance of long filamentous rods with very few motile cells. These observations provide direct evidence that many types of micro-organisms are present in sludge.

Additional evidence was provided by culture counts on dilution series in which glucose or cellobiose was the fermentable substrate.

With glucose a count of  $10^9$  bacteria per ml was obtained and a parallel series containing cellobiose showed  $10^8$  bacteria. In view of these numerous additional bacteria, the present results cannot be considered to give a complete picture of the sludge fermentation but they do provide information on an important part of the process.

### S u m m a r y.

Analyses of sewage solids show cellulose to be one of the chief components. Culture counts of cellulolytic bacteria in a primary anaerobic sewage digester show them to be present in numbers as high as 1 million per ml. The tendency of cellulolytic bacteria to cling to cellulose fibers makes it highly probable that the number of cellulolytic cells is much larger.

All 10 cellulolytic strains isolated in pure culture show better growth in solid than in liquid media, and for some of them agar possesses growth promoting properties. For some strains, phytone and trypticase can replace the agar but other strains could not be grown in media containing no agar.

Hydrogen, carbon dioxide, ethanol, formic acid, acetic acid, and lactic acid have been identified as fermentation products and glucose shown to be a product of cellulose digestion. Cellobiose, starch, dextrin, and maltose were fermented by 5 tested strains, inulin and esculin by one of them, but none of 17 other carbohydrates, including glucose, were attacked.

The rate of cellulose fermentation by a mixed culture of a *Clostridium* sp. and a cellulose decomposer is much greater than the rate by the latter alone. The rate of fermentation by a pure culture is not affected by acetate concentrations up to 5000 parts per million. It is postulated that the rate of fermentation of cellulose may be the factor limiting the rate of sewage fermentation though more evidence regarding rates of fermentation of other constituents of sewage is needed before final conclusions can be drawn.

### R e f e r e n c e s.

1. BARKER, H. A. 1940. *Antonie van Leeuwenhoek* **6**, 201-220.
2. BARKER, H. A. 1943. *Proc. Nat. Acad. Sci. U.S.* **29**, 184-190.
3. BUSWELL, A. M. 1936. *State of Illinois Division of the State Water Survey, Bull.* **32**.
4. HORROCKS, R. H. and MANNING, G. B. 1949. *Lancet* **256**, 1042-1045.

5. HUNGATE, R. E. 1950. *Bact. Rev.* **14**, 1-49.
  6. LEETE, G. V. 1952. *Proc. Fourth Ann. Pacific Northwest Industrial Waste Conf.* p. 91-93, Washington State College.
  7. MCBEE, R. H. 1948. *J. Bact.* **56**, 653-663.
  8. MYLROIE, R. L. 1953. Thesis. Washington State College.
  9. PARTRIDGE, S. M. 1946. *Nature* **158**, 270-271.
  10. SCHNELLEN, CH. G. T. P. 1947. *Onderzoekingen over de Methaangisting*. Thesis Delft.
  11. SÖHNGEN, N. L. 1910. *Rec. trav. chim.* **29**, 238-274.
-

(From the Laboratory of Tropical Hygiene, Institute of Tropical Medicine,  
University of Leiden).

## CHEMOTHERAPY OF CARCINOMA IMPROVED BY HOST CONDITIONING

### II. LOCAL CHEMOTHERAPY COMBINED WITH INDIRECT TREATMENT<sup>1)</sup>

by

**A. G. AUDIER**

(Received August 15, 1953).

#### INTRODUCTION.

A case of malignant tumor always has the best prognosis when radical, local therapy is possible. The problem, however, is that radical therapy is often impossible, and that in some cases metastases are found, after a "radical" operation, in places where no metastases existed previously. The possibility must be taken into account that the apparently healthy tissue surrounding a malignant tumor is no longer quite normal, but contains precancerous, and perhaps even cancerous cells (DRUCKREY, 1951).

Only in relatively rare cases can malignant tumors be cured only by operation, irradiation, or medication. The possible value of different combined therapies has frequently been investigated. In most cases, operation was combined only with local irradiation, while — apart from the treatment of certain tumors with hormones — chemotherapy was regarded as being indicated in only a limited number of cases. This is understandable, because most substances which would be eligible chemotherapeutically, inhibit mitosis, and also damage the normal body-cells, while the more physiological mitosis-inhibiting substances such as choline (FECHT, 1949) are usually ineffective once a tumor has been formed.

Treatment with the aid of reactions of the general adaptation system (G.A.S), combined with host-conditioning, yielded satis-

---

<sup>1)</sup> Part I: *Antonie van Leeuwenhoek* **19**, 83, 1953.



factory results, both in animal experiments (AUDIER, 1953) and when tried clinically (ISSELS, 1953).

Part I of the present communication contains an account of the experimental inoculation of mice with tumor-material, which was at first followed by tumor formation, but subsequently by necrosis and shedding of the tumor. When the tumorous material and the bacteria were given mixed together, the tumor was shed in 100% of cases, 50% of the test-animals recovering permanently.

Further experiments showed that these figures fell to 50 and 38%, respectively, when the tumorous material and the bacterial product, although injected simultaneously, were separated locally. In none of the experiments did indirect treatment via the general adaptation system equal the results of the direct local therapy.

These experiments suggested:

1. that one and the same therapeutic agent can act, simultaneously, both locally and via the G.A.S.;
2. that, however, local chemotherapy is more active than indirect therapy via the G.A.S.

These considerations suggested that it should be possible to apply a local chemotherapy which,

- a) would destroy the tumor-cells and the precancerous-cells in and around the tumor;
- b) would attack as well as prevent metastases.

In contrast to what has been found after operations, this local treatment would not increase, but reduce, the danger of relapse and metastasis, while at the same time any metastases, already present elsewhere, would also be attacked. And in contrast to the treatment with mitosis-inhibiting agents, it would now be possible to prevent general cellular damage, especially if Shwarzman's therapy were combined with hormonal products.

In addition, treatment via the Shwarzman phenomenon would in some cases have another special advantage. For, whereas local treatment is usually successful only in the case of fairly small tumors, it is well known that the Shwarzman phenomenon actually intensifies when the size of the tumor is large: "The intensity of the phenomenon seemed to bear a direct relationship to the age and size of the tumor. While very young perfectly healthy tumors gave no reaction, larger tumors gave practically 100% positive results" (SHWARZMAN, 1937).

It accordingly seemed that a local chemotherapy, combined with reactions of the G.A.S., might offer particular advantages in medical

practice, and that an investigation into the value of such a therapy might yield important results.

## EXPERIMENTS.

### 1. Product used.

The first thing sought after was a chemotherapeutic agent which would be more suitable for local treatment than bacteria or bacterial products. The following considerations led to try a combined treatment with yeast-extract:

- a) PROTTI (1950) succeeded in causing certain tumor-cells to degenerate selectively, *in vitro*, with the aid of certain yeasts.
- b) GOTTSCHALK (1951) provoked curative reactions in human tumors by intravenous injections of PROTTI's yeasts.
- c) LEWISOHN and co-workers (1941), by intravenous injections of laboratory animals with yeast-extract, caused the disappearance of spontaneous tumors.
- d) BOCK (1932) succeeded in provoking a Shwarzman phenomenon by filtrates of yeast cultures.
- e) IVÁNOVICS (1934) caused Shwarzman reactions with the aid of yeast-extract.

The object of the experiments, therefore, was to discover whether treatment with yeast-extract could be applied successfully to malignant tumors. It should become evident whether the local therapy in itself would be able to cause sufficient reactions in the G.A.S., or whether it would be necessary to give intravenous injections so as to get a sufficiently intensive Shwarzman phenomenon. Finally, I should have to ascertain whether a combination of this therapy with either operation or irradiation would be possible or desirable.

### 2. Experiments on inoculated tumors in mice, with yeast-extract.

To have the greatest possible chance of success, use was made of the yeasts discovered by PROTTI, and kindly placed by him at my disposal. The extract was prepared according to the method of LEWISOHN and co-workers.

## A. Local therapy.

With a view to the local therapy the following tests were made: In the first place it was ascertained where the extract should

TABLE I.  
Local intra- and peri-tumoral administration <sup>1)</sup> of an  
extract from yeast E. 14 (pH 5.5).

|               | Mouse<br>No.  | No. of<br>injec-<br>tions | start of<br>thera-<br>py <sup>2)</sup> | tumor<br>smaller | tumor<br>disap-<br>peared | dead | still<br>alive | Particulars                                                            |
|---------------|---------------|---------------------------|----------------------------------------|------------------|---------------------------|------|----------------|------------------------------------------------------------------------|
|               |               |                           | No. of days after inoculation          |                  |                           |      |                |                                                                        |
| Controls      | C1, C2,       |                           |                                        |                  |                           | 16   | —              | very large tumor                                                       |
|               | C3,<br>C4, C5 | —<br>—                    | —<br>—                                 | —<br>—           | —<br>—                    | 34   | —              | very large tumor                                                       |
| Intra-tumoral | 167           | 18                        | 9                                      | —                | —                         | 30   | —              | very large tumor                                                       |
|               | 168           | 17                        | 9                                      | —                | —                         | 30   | —              | small tumor                                                            |
|               | 176           | 20                        | 7                                      | —                | —                         | 31   | —              | very large tumor                                                       |
|               | 177           | 20                        | 7                                      | —                | —                         | 30   | —              | large tumor                                                            |
|               | 179           | 18                        | 7                                      | —                | —                         | 25   | —              | large tumor                                                            |
| Peri-tumoral  | 162           | 8                         | 9                                      | 13               | —                         | 20   | —              | death due to flaw<br>in treatment;<br>tumor, hard<br>coat, soft center |
|               | 169           | 6                         | 8                                      | —                | 10                        | —    | 100            | tumor disappea-<br>red                                                 |
|               | 178           | 5                         | 7                                      | —                | 12                        | —    | 78             | tumor disappea-<br>red                                                 |
|               | 181           | 24                        | 7                                      | 21               | —                         | —    | 78             | small hollow tu-<br>mor, hard skin<br>only                             |

<sup>1)</sup> During the injection therapy an extract of *Glycyrrhiza glabra* was given per os daily. cf. Part I: Antonie van Leeuwenhoek **19**, 83, 1953.

<sup>2)</sup> In all experiments, the tumor had the size of a small pea at the beginning of the treatment. The experiments shown in Table V form an exception to this.

be injected, i.e. whether inside the tumor or around its outer margin (Table I).

The action of two extracts from two different yeasts, i.e. E. 14 and E. VIII, was then compared (Table II).

In order to obviate difficulties in the case of clinical application, it seemed desirable to use a product with a pH 7.0. Part of the extract was therefore brought to pH 7.0, and its action compared with that of the same extract with pH 5.5 (Table III).

Since, for a therapy with Shwarzman reactions, an extract of

TABLE II.

Local peri-tumoral administration of an extract of yeast  
E. 14 and E. VIII (pH 5.5)<sup>1)</sup>.

|                 | Mouse<br>No. | No of<br>injec-<br>tions | start of<br>thera-<br>py <sup>2)</sup> | tumor<br>smaller | tumor<br>disap-<br>peared | dead | still<br>alive | Particulars                        |
|-----------------|--------------|--------------------------|----------------------------------------|------------------|---------------------------|------|----------------|------------------------------------|
|                 |              |                          | No. of days after inoculation          |                  |                           |      |                |                                    |
| Extract E. 14   | 221          | 9                        | 6                                      | 14               | —                         | —    | 85             | tumor: a couple<br>of small points |
|                 | 182          | 6                        | 7                                      | —                | 10                        | —    | 80             | tumor disap-<br>peared             |
|                 | 183          | 4                        | 7                                      | —                | —                         | 12   | —              | small tumor                        |
|                 | 184          | 4                        | 7                                      | —                | 14                        | —    | 83             | tumor disap-<br>peared             |
| Extract E. VIII | 188          | 3                        | 9                                      | —                | —                         | 16   | —              | large tumor                        |
|                 | 192          | 4                        | 9                                      | —                | 16                        | —    | 74             | tumor disap-<br>peared             |
|                 | 195          | 3                        | 9                                      | —                | 16                        | —    | 74             | tumor disap-<br>peared             |
|                 | 196          | 3                        | 8                                      | —                | 10                        | —    | 74             | tumor disap-<br>peared             |

<sup>1)</sup> <sup>2)</sup> see footnotes <sup>1)</sup> <sup>2)</sup> Table I.

TABLE III.

Local peri-tumoral administration of an extract of yeast E. VIII  
with pH 5.5 and 7.0<sup>1)</sup>.

|        | Mouse<br>No. | No. of<br>injec-<br>tions | start of<br>thera-<br>py <sup>2)</sup> | tumor<br>smaller | tumor<br>disap-<br>peared | dead | still<br>alive | Particulars            |
|--------|--------------|---------------------------|----------------------------------------|------------------|---------------------------|------|----------------|------------------------|
|        |              |                           |                                        |                  |                           |      |                |                        |
|        |              |                           |                                        |                  |                           |      |                |                        |
| pH 5.5 | 239          | 3                         | 9                                      | 13               | —                         | 14   | —              | very small tumor       |
|        | 240          | 14                        | 9                                      | —                | 25                        | —    | 74             | tumor disap-<br>peared |
|        | 242          | 5                         | 9                                      | —                | 16                        | —    | 74             | tumor disapp.          |
|        | 243          | 5                         | 9                                      | —                | 18                        | —    | 74             | tumor disapp.          |
|        | 246          | 5                         | 8                                      | —                | 10                        | —    | 74             | tumor disapp.          |
| pH 7.0 | 194          | 4                         | 9                                      | —                | —                         | 10   | —              | large tumor            |
|        | 197          | 3                         | 8                                      | —                | —                         | 12   | —              | large tumor            |
|        | 198          | 8                         | 8                                      | —                | —                         | 18   | —              | large tumor            |
|        | 199          | 16                        | 8                                      | —                | —                         | 27   | —              | large tumor            |
|        | 203          | 12                        | 8                                      | —                | —                         | 20   | —              | large tumor            |
|        | 205          | 10                        | 8                                      | —                | —                         | 20   | —              | large tumor            |

<sup>1)</sup> <sup>2)</sup> see footnotes <sup>1)</sup> <sup>2)</sup> Table I.

TABLE IV.

Local peri-tumoral administration of an extract of yeast E. 14 (pH 5.5) with and without simultaneous administration per os of an extract of *Glycyrrhiza glabra*.

| Mouse No. | <i>Glycyrrhiza glabra</i> -extract per os | No. of injections | start of therapy 1)           | tumor smaller | tumor disappeared | dead | shock dead | still alive | Particulars                           |
|-----------|-------------------------------------------|-------------------|-------------------------------|---------------|-------------------|------|------------|-------------|---------------------------------------|
|           |                                           |                   | No. of days after inoculation |               |                   |      |            |             |                                       |
| 212       | +                                         | 9                 | 9                             | 13            | —                 | 20   | —          | —           | small tumor                           |
| 169       | +                                         | 6                 | 9                             | —             | 10                | —    | —          | 100         | tumor disappeared                     |
| 228       | +                                         | 5                 | 7                             | —             | 12                | —    | —          | 78          | tumor disappeared                     |
| 231       | +                                         | 24                | 7                             | —             | 31                | —    | —          | 78          | tumor disappeared                     |
| 238       | +                                         | 6                 | 9                             | —             | —                 | 16   | —          | —           | large tumor                           |
| 232       | —                                         | 12                | 7                             | 12            | —                 | 21   | +          | —           | small tumor                           |
| 233       | —                                         | 3                 | 7                             | —             | —                 | 10   | +          | —           | small tumor                           |
| 234       | —                                         | 4                 | 7                             | —             | 14                | —    | —          | 74          | tumor disappeared                     |
| 189       | —                                         | 3                 | 9                             | 13            | —                 | 14   | +          | —           | small tumor                           |
| 190       | —                                         | 14                | 9                             | 21            | —                 | 25   | +          | —           | large tumor, only slightly diminished |
| 223       | —                                         | 10                | 8                             | 13            | —                 | 20   | +          | —           | small tumor                           |
| 225       | —                                         | 10                | 8                             | 17            | —                 | 20   | +          | —           | large tumor, only slightly diminished |

1) See footnote 2) Table I.

TABLE V.

Local peri-tumoral administration of an extract of yeast E. 14, either alternated or not with intravenous injections of the same yeast-extract, in the case of large tumors 1), with simultaneous administration of an extract of *Glycyrrhiza glabra* per os.

| Mouse No. | No. of local injections | No. of intra-venous injections | start of therapy | tumor smaller | tumor disap-peared | dead | still a live | Particulars                     |
|-----------|-------------------------|--------------------------------|------------------|---------------|--------------------|------|--------------|---------------------------------|
|           |                         |                                |                  |               |                    |      |              |                                 |
| 213       | 6                       | 5                              | 12               | 14            | —                  | —    | 70           | small hard tumor                |
| 214       | 10                      | 8                              | 14               | 10            | —                  | —    | 74           | very small, hard tumor          |
| 215       | 6                       | 4                              | 12               | —             | 10                 | —    | 76           | tumor disappeared               |
| 216       | 20                      | 10                             | 16               | —             | —                  | 30   | —            | small tumor                     |
| 217       | 10                      | —                              | 12               | 16            | —                  | 20   | —            | renewed growth into large tumor |
| 218       | 8                       | —                              | 14               | —             | —                  | 24   | —            | large tumor                     |
| 219       | 6                       | —                              | 12               | —             | —                  | 30   | —            | large tumor                     |
| 220       | 14                      | —                              | 13               | 18            | —                  | 34   | —            | renewed growth into large tumor |

1) 1 — 1½ cm diameter.



*Glycyrrhiza glabra* was indispensable (AUDIER, 1953), I wished to ascertain the effect of this extract when administered per os, simultaneously with the peri-tumoral injections of yeast-extract (Table IV).

## B General and combined therapy.

### a) Intravenous injections of yeast-extract.

The same yeast-extract used for the peri-tumoral therapy was injected intravenously. At first this was done in the dosage indicated by LEWISOHN; with the invariable result, however, of the test-animal's death after the second or third injection, coupled with shock phenomena, notwithstanding the simultaneous administration of an extract of *Glycyrrhiza glabra*. In a dosage ten times lower than LEWISOHN's, the yeast-extract was well tolerated, and followed by necroses in the tumors.

Owing to considerable technical difficulties experienced with repeated intravenous injections in mice we continued our experiments with rabbits, which had been inoculated with the Brown-Pearce tumor. It proved possible to cause also the Brown-Pearce tumors to disappear by means of intravenous injections of an extract of yeast E VIII. These experiments will be described in a subsequent publication.

### b) Combined treatment.

Experiments were then made with a combined peri-tumoral and intravenous treatment. For these experiments, mice were used with large tumors which had hardly, or not at all, responded to the local therapy alone. The treatment was alternately local and intravenous, i.e. one day local, the next day intravenous, and so on. As Table V shows, this combined treatment actually appears to give better ultimate results than the local treatment alone.

## RESULTS.

A. Inoculated tumors on mice proved fatal in 16 and 34 days, respectively (Table I : C1, C2, C3, C4, C5).

B. Local intra- and peri-tumoral administration of an extract of yeast E. 14 (pH 5.5).

1. Intra-tumoral injections had little or no effect on the tumors (Table I: Nos. 167, 168, 176, 177, 179).

2. Peri-tumoral injections caused the tumors to decrease and/or disappear (Table I: Nos. 162, 169, 178, 181).

- C. Local peri-tumoral administration of an extract of two different yeasts, i.e. E. 14 and E. VIII, pH 5.5 proved both to be active (Table II).
- D. Local peri-tumoral administration of an extract of yeast E. VIII with pH 5.5 and 7.0.
  - 1. The extract with pH 5.5 had a distinct effect on the tumors in every case (Table III: Nos. 239, 240, 242, 243, 246).
  - 2. The extract with pH 7.0 had no effect in any of the cases treated (Table III: Nos. 194, 197, 198, 199, 203, 205).
- E. Local peri-tumoral administration of an extract of yeast E. 14 (pH 5.5) with and without simultaneous administration per os of an extract of *Glycyrrhiza glabra*.
  - 1. No shock conditions were observed in the mice treated with *Glycyrrhiza glabra* in the dosage used (Table IV: Nos. 212, 169, 228, 231, 238).
  - 2. Out of the seven mice treated in the same way, but without *Glycyrrhiza glabra*, six suffered from shock resulting in death (Table IV: Nos. 232, 233, 189, 190, 223, 225).
- F. Local peri-tumoral administration of an extract of yeast E. 14, either alternated or not with intravenous injections of the same yeast-extract in the cases of large tumors, with simultaneous administration of an extract of *Glycyrrhiza glabra* per os. With the combined therapy the results were better (Table V: Nos. 213, 214, 215, 216) than with the local therapy alone (Table V: Nos. 217, 218, 219, 220).

#### CONCLUSION.

Although the number of test-animals experimented with was — as in part I of the present investigation — relatively small, preliminary conclusions may be justified, since the development of all tumors, once started was fatal without therapy.

In conclusion we may say that the yeast-extract proved suitable for the local treatment of malignant tumors. To obtain efficient action the extract must have an acid pH and be injected around the outer margin of the tumor. Combination with an extract of *Glycyrrhiza glabra* per os proved desirable.

On the basis of the experiments, it may be assumed that local and combined chemotherapy of malignant tumors opens new perspectives, and that extract of yeast will probably prove to be an important aid in this respect.

As a matter of fact, some clinical trials were already made in the course of this investigation, under the direction of G. KORT-HOF, of the Surgical Department, Academic Hospital, Leyden. The results of these trials, which open further perspectives in regard to the way of action of the yeast-extract and its accompanying phenomena, will form the subject of a further communication yet to be published.

### S u m m a r y.

Inoculated tumors in mice were successfully treated with yeast-extract, with simultaneous administration of an extract of *Glycyrrhiza glabra* per os. The therapy was improved by alternating the local treatment with intravenous injections of the same yeast-extract.

### A c k n o w l e d g e m e n t s.

I wish to express my indebtedness to Professor Dr J. E. DINGER of the University of Leyden, for his constant encouragement and constructive criticism during these investigations.

I also wish to acknowledge the kindness of Dr G. PROTTI, Busto Arsizio, Italy, who generously supplied his yeast cultures.

Further acknowledgement is made to the Royal Netherlands Yeast and Spirit Works, Delft, for the cultivation of the yeast and the preparation of the extracts.

### L i t e r a t u r e.

- AUDIER, A. G. 1953. *Antonie van Leeuwenhoek* **19**, 83.  
 BOCK, H. 1932. *Zentralbl. f. Bakt. I.* **125**, 1, 435.  
 DRUCKREY, H. 1951. *Arzneimittelforschung* **1**, 383.  
 FECHT, K. E. 1949. *Strahlentherapie* **80**, 95.  
 GOTTSCHALK, F. 1951. *Münch. Med. Wschr.* **93**, 1708.  
 ISSELS, J. 1953. *Grundlagen und Richtlinien für eine interne Krebstherapie.* Marquard & Cie, Stuttgart.  
 IVÁNOVICS, G. 1934. *Magyar-orvosi Arch.* **35**, 362.  
 LEWISOHN, R., LEUCHTENBERGER, C., LEUCHTENBERGER, R., LASZO, D. and BLOCK, K. 1941. *Cancer Research* **1**, 799.  
 PROTTI, G. 1950. *Tumori* **36**, 14.  
 SELEY, H., 1950. *The physiology and pathology of exposure to stress.* Acta Inc., Montreal.  
 SHWARZMAN, G. 1937. *Phenomenon of local tissue reactivity.* Paul B. Hoeber Inc., New York.
-

(Laboratoire de Microbiologie, Université de Louvain, Héverlé-lez-Louvain, Belgique).

## ÉTUDE DU MÉTABOLISME D' ACIDES AMINÉS CHEZ *ASPERGELLUS ORYZAE*

### IV. INFLUENCE DU CHLORURE DE CALCIUM SUR LE MÉTABOLISME DES ACIDES AMINÉS<sup>1)</sup>

par

PAUL SIMONART et KWANG YÜ CHOW

(Reçu le 25 Juillet 1953).

Il a été montré que le calcium active et stabilise les protéinases bactériennes (1, 2, 3, 4) et que cet élément est nécessaire au développement de bactéries dans un milieu qui ne contient que des protéines comme seule source d'azote (5).

Dans le domaine des moisissures CHOW (6) a mis en évidence l'influence favorable du calcium sur le développement d'*Aspergillus oryzae* cultivé sur hydrolysate de caséine, ainsi que sur l'activité protéolytique de la protéinase de cette moisissure. D'autre part, SAKAGUCHI (7) a observé une augmentation de teneur en azote d'*Asp. oryzae* en présence de carbonate calcique.

Se basant sur ces observations et faisant suite à une étude des acides aminés présents à l'état libre dans le mycélium d'*Asp. oryzae* (8, 9), il a paru intéressant de rechercher si le calcium exerçait une influence sur le métabolisme des acides aminés de ce microchampignon. Il en résulte qu'en effet une différence sensible de composition d'acides aminés libres s'observe chez cette moisissure suivant qu'elle est cultivée en présence ou en absence de calcium.

#### I. MÉTHODES EXPÉRIMENTALES.

1) La souche de moisissure et le milieu de culture utilisés dans

---

<sup>1)</sup> Part I, II, III: Antonie van Leeuwenhoek **19**, 121, 245, 1953; **20**, 174, 1954.

cette étude sont ceux qui ont servi aux recherches précédentes (8, 9): *Asp. oryzae* w.f. et une solution à 3 % de bacto-casamino acids (Difco). Mais plutôt que de se faire par la méthode sur flux continu de milieu, les cultures se font ici en erlenmeyers de 100 ml contenant 20 ml de liquide nutritif que l'onensemence au fil de platine au moyen d'une culture sur moût-agar, vieille de trois jours. L'erlenmeyerensemencé est maintenu à 25° C., habituellement pendant cinq jours.

2) Pour l'analyse des acides aminés libres, on retire le mycélium de l'erlenmeyer, et après trois lavages à l'eau on en extrait les substances solubles par ébullition de deux minutes dans 20 ml d'eau. On concentre cet extrait sous pression réduite à  $\pm 45^{\circ}$  C. et le soumet à un examen chromatographique selon les méthodes appliquées pour les études précédentes. Cependant, dans cette recherche-ci, il est utile de débarrasser la solution à chromatographier des sels ainsi que des substances acides et neutres qu'elle peut contenir. C'est pourquoi il est fait usage de la façon suivante d'une colonne de  $20 \times 1$  cm de résine ionique "Zeo- Karb". On lave la colonne avec 100 ml de NaOH 2 N, puis à l'eau jusqu'à neutralité de l'éluat, ensuite avec 100 ml d'HCl 2 N, enfin à l'eau jusqu'à ce que l'éluat ait un  $\text{pH} = 5$ . On verse alors sur la colonne 0,5 à 1 ml de la solution à étudier, on la laisse s'écouler doucement, puis on y ajoute deux fois 1 ml d'eau, enfin on élue à l'eau et recueille environ 20 gouttes par minute jusqu'à ce que le  $\text{pH}$  de l'éluat atteigne 5. Après cela on élue les acides aminés à l' $\text{NH}_4\text{OH}$  1,5 N, recueillant deux fractions d'éluat, la première de 100 à 150 ml et la seconde de 50 ml; on les concentre sous pression réduite et les analyse chromatographiquement. On ne soumet cependant la seconde fraction à l'analyse que si des acides aminés basiques sont présents.

## II. RÉSULTATS ET DISCUSSION.

### 1. Influence du $\text{CaCl}_2$ sur la croissance d'*Asp. oryzae*.

*Asp. oryzae* se développe très médiocrement sur 3 % de casamino acids. Mais l'addition d'une quantité convenable de calcium favorise très fort le développement de la moisissure ainsi que le montre le tableau 1. Celui-ci donne le poids du mycélium formé en 5 jours à 25° C. sur 20 ml de milieu, en erlenmeyer de 100 ml, et il montre qu'une concentration M/50 de  $\text{CaCl}_2$  donne une pellicule mucédienne



qui pèse au delà de six fois plus que celle qui est formée en absence de ce sel. D'autre part, ce tableau indique aussi le pH de la solution de métabolisme à la fin de la culture de cinq jours, et fait ressortir que non seulement le pH le plus bas correspond au développement le meilleur, mais que d'une culture à l'autre il varie en sens inverse du poids du mycélium.

TABLEAU I.

|                                                           | 3% bacto-casamino acids; pH = 6.3 |                             |                            |                            |                            |
|-----------------------------------------------------------|-----------------------------------|-----------------------------|----------------------------|----------------------------|----------------------------|
|                                                           |                                   | +M/100<br>CaCl <sub>2</sub> | +M/50<br>CaCl <sub>2</sub> | +M/20<br>CaCl <sub>2</sub> | +M/10<br>CaCl <sub>2</sub> |
| Poids du mycélium<br>séché à 105° C.<br>pendant 12 heures | 0.020 g                           | 0.035 g                     | 0.124 g                    | 0.101 g                    | 0.084 g                    |
| pH de la solution de<br>métabolisme                       | 8.2                               | 7.9                         | 6.5                        | 7.0                        | 7.1                        |

## 2. Influence du chlorure de calcium sur la teneur en acides aminés de la solution de métabolisme.

Le chromatogramme du milieu de culture tel quel (3 % bacto-casamino acids (Difco)) montre les spots correspondants aux substances suivantes: acide aspartique, acide glutamique, sérine, glycine, thréonine, alanine, histidine, lysine, proline, arginine, phényl-alanine et des acides aminés du groupe valine et leucine.

Si l'on chromatographie le même milieu de culture additionné de chlorure de calcium, ne fut-ce qu'à la concentration de M/100, on y décèle les mêmes acides aminés qu'en absence de ce sel, sauf pour les acides aminés basiques histidine, lysine et arginine qu'il ne renferme pas.

Ceci s'explique par l'insolubilisation de ces substances sous forme de sels calciques. En effet, lors de l'addition de chlorure de calcium se forme un précipité qui fut analysé comme suit: après filtration et lavages répétés à l'eau on le dissout dans H<sub>2</sub>SO<sub>4</sub> 0.5 N; on élimine par filtration le sulfate de calcium; on passe le filtrat à travers une colonne de "Zeo Karb", et recueille l'éluat ammoniacal; la chromatographie de celui-ci montre la présence de lysine à côté de petites quantités d'arginine et d'histidine.

La composition qualitative en acides aminés de ces milieux de culture, avec ou sans chlorure de calcium ne se modifie guère lors du développement d'*Asp. oryzae* pendant cinq jours. La seule différence perceptible est que l'on trouve parfois de l'ornithine, de la glutamine et de la lysine dans la solution de métabolisme calcique.

### 3. Influence du chlorure de calcium sur la teneur en acides aminés libres du mycélium.

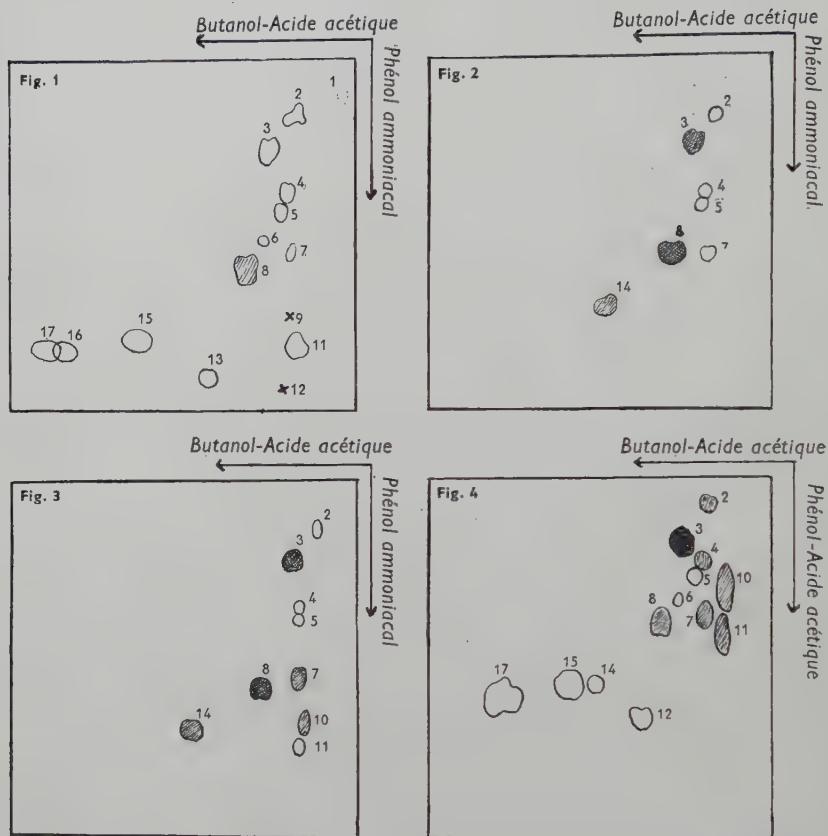
Dans le mycélium formé sur le milieu exempt de chlorure de calcium on retrouve chromatographiquement (fig. 1) toutes les substances aminées du bacto-casamino acids, à côté de traces de glutamine et de cystéine ou de cystine.

Lorsque la moisissure se développe sur 3 % de bacto-casamino acids contenant du chlorure de calcium à la concentration M/100 on ne décèle chromatographiquement (fig. 2) dans son mycélium que l'acide aspartique, l'acide glutamique, la sérine, la glycine et l'alanine, en tant que constituants initialement présents dans le milieu de culture. Mais en plus de ces cinq acides aminés on trouve encore en quantité appréciable l'acide  $\gamma$ -aminobutyrique et la glutamine.

L'absence de proline, de valine et de leucine peut s'expliquer soit par une inhibition d'absorption de ces acides par le calcium, soit par leur décomposition plus rapide en présence de cet élément. Les seules données qualitatives dont on dispose ici ne permettent pas de dire à quel phénomène on a affaire. Des déterminations quantitatives trancheraient la question.

Quant on cultive la moisissure sur un milieu plus riche en calcium telles des concentrations en chlorure de calcium de M/50, de M/20 et de M/10, la teneur en substances aminées se distingue de celle qui vient d'être vue pour la moisissure cultivée avec moins de chlorure de calcium. En effet (fig. 3 et 4) on y décèle de l'ornithine, de la lysine et des traces d'arginine qu'on ne trouve pas en absence de calcium ni à la concentration M/100 de chlorure de calcium. En outre, à la concentration M/20 on ne trouve plus que des traces d'acide  $\gamma$ -aminobutyrique, tandis qu'à la concentration M/10 cet acide ne peut plus être mis en évidence. Enfin, il semble que la plus forte concentration en ornithine corresponde à la concentration M/20.

Ces résultats appellent quelques observations supplémentaires en ce qui concerne la formation d'acide  $\gamma$ -aminobutyrique et celle d'acides aminés basiques.



Chromatogrammes des acides aminés présents à l'état libre dans le mycélium d'*Aspergillus oryzae*.

Fig. 1: culture sur 3% de bacto-casamino acids sans chlorure de calcium;

Fig. 2: culture sur 3% de bacto-casamino acids contenant M/100 de chlorure de calcium;

Fig. 3: culture sur 3% de bacto-casamino acids contenant M/50 de chlorure de calcium;

Fig. 4: culture sur 3% de bacto-casamino acids contenant M/20 de chlorure de calcium.

1 = cystéine; 2 = acide aspartique; 3 = acide glutamique; 4 = sérine; 5 = glycine; 6 = thréonine; 7 = glutamine; 8 = alanine; 9 = histidine; 10 = ornithine; 11 = lysine; 12 = arginine; 13 = proline; 14 = acide  $\gamma$ -aminobutyrique; 15 = valine et méthionine; 16 = phénylalanine; 17 = leucine.

Pour ce qui est de l'acide  $\gamma$ -aminobutyrique, il est intéressant de noter que la présence de cet acide dans le mycélium s'observe d'une part, lorsque la moisissure est cultivée sur milieu contenant M/100 à M/20 de chlorure de calcium et d'autre part lorsqu'elle est cultivée sur "flux continu" à pH bas (8). L'acidité et le calcium semblent donc exercer — probablement par un mécanisme différent — une action analogue sur le métabolisme des acides aminés. Alors que l'on estimait que la production d'acide  $\gamma$ -aminobutyrique ne se faisait qu'à pH bas, l'on constate ici qu'elle peut avoir lieu à pH relativement élevé en présence d'une quantité convenable de chlorure de calcium. Des résultats analogues ont été obtenus en présence de carbonate calcique.

Quant aux acides aminés basiques, ornithine, lysine et arginine que l'on décèle dans le mycélium développé en présence de quantités suffisantes de chlorure de calcium, ils ne peuvent provenir que de modifications biochimiques d'autres acides aminés, favorisées par le calcium. En effet, d'une part, ce milieu de culture ne renferme aucune de ces trois substances en quantités décétables, d'autre part, le mycélium formé en absence de calcium ne les contient pas davantage.

Comme ces acides aminés basiques trouvent probablement leur origine dans les réactions couplées de désamination et d'amination, cela revient à dire que, plutôt que de donner lieu à la mise en liberté d'ammoniaque, la désamination de tel ou tel acide aminé produirait — directement ou indirectement — des acides bi- ou pluri-aminés. Dans ces conditions le milieu ne deviendra pas aussi alcalin que si l'on avait affaire à des désaminations simples. Et il est plausible d'admettre que le rôle essentiel du calcium — et peut être aussi dans une certaine mesure celui de l'acidité — dans le métabolisme qui est étudié ici, se ramène surtout à exercer indirectement une action tampon.

Cette hypothèse se confirme encore par le fait que l'acide  $\gamma$ -aminobutyrique et la glutamine prennent aussi naissance dans le mycélium de la moisissure lorsque le milieu contient une quantité convenable de calcium (ou qu'il a un pH bas). Or, d'une part, la décarboxylation de l'acide glutamique avec formation d'acide  $\gamma$ -aminobutyrique désacidifie le milieu, en supprimant un groupement carboxyle et d'autre part, l'amidation de l'acide glutamique bloque aussi un groupe carboxyle en même temps qu'elle évite la mise en liberté d'une molécule d'ammoniaque.

On constate donc que par formation d'acides aminés basiques et d'amide ou encore accessoirement par décarboxylation, le calcium agit à la façon d'un tampon. Mais il n'est pas établi si cet élément favorise des phénomènes de transamination ou bien s'il inhibe les désaminations simples. Cette action tampon permet encore d'expliquer l'influence du calcium dans d'autres phases du métabolisme de la cellule. Mais cette hypothèse n'exclut néanmoins pas qu'un rôle particulier puisse être joué par l'acide  $\gamma$ -aminobutyrique.

### R é s u m é.

La teneur en acides aminés libres du mycélium est différente selon que l'on cultive *Aspergillus oryzae* sur 3 % de bacto-casamino acids tel quel ou sur ce milieu additionné de chlorure de calcium.

Pour des concentrations M/100 de  $\text{CaCl}_2$  on y décèle chromatographiquement en quantité appréciable l'acide  $\gamma$ -aminobutyrique et la glutamine que l'on ne trouve pas en absence de ce sel. Tandis que pour des concentrations M/50 on y met encore en évidence l'ornithine, la lysine et l'arginine que l'on ne décèle pas pour des concentrations plus faibles de chlorure de calcium.

Ces résultats suggèrent que le calcium déclenche indirectement une action tampon en favorisant d'une part la formation d'acides aminés basiques et d'autre part la décarboxylation de l'acide glutamique avec production d'acide  $\gamma$ -aminobutyrique.

Il est à remarquer que cette dernière réaction, en présence de chlorure de calcium, se produit à pH élevé, alors qu'il est généralement admis que cette décarboxylation ne se fait qu'à pH bas.

### Bibliographie.

1. BEUMER, J. 1941. Acta Biol. Belg. **2**, 273, 276.
2. GORINI, L. et FROMAGEOT, C. 1949. Compt. Rend. **229**, 559.
3. GORINI, L. et FROMAGEOT, C. 1950. Biochim. Biophys. Acta **5**, 524.
4. GORINI, L. 1950. Biochim. Biophys. Acta **6**, 237.
5. GORINI, L. et ANDRAIN, L. 1951. Biochim. Biophys. Acta **6**, 477.
6. CHOW, K. Y. 1951. Enzymes d'*Aspergillus* et biochimie de la fermentation du Soja. Thèse doct., Univ. Louvain.
7. SAKAGUCHI, K. 1950. J. Agr. Chem. Soc. Japan **24**, 77.
8. SIMONART, P. et CHOW, K. Y. 1953. Antonie van Leeuwenhoek **19**, 121, 245.
9. SIMONART, P. et CHOW, K. Y. 1954. Antonie van Leeuwenhoek **20**, 174.



(From the Department of Food Technology, University of California,  
Berkeley, California).

## CAROTENOIDS IN ASPOROGENOUS YEASTS

by

**T. NAKAYAMA, G. MACKINNEY and H. J. PHAFF**<sup>1)</sup>

(Received August 14, 1953).

Because sexual fruiting bodies are absent in the Fungi Imperfecti, the classification cannot depend primarily on phylogenetic considerations but must be based to a large extent on biochemical and morphological differences. In the recent handbook on yeast classification by the Dutch School (13) the asporogenous yeasts are placed in one family, the *Cryptococcaceae*, which is subdivided into three subfamilies, the *Cryptococcoideae*, the *Trichosporoideae* and the *Rhodotoruloideae*. They recognize the last-mentioned, containing the single genus *Rhodotorula*, by its color (yellow to red) due to the presence of carotenoid pigments. The subfamily *Cryptococcoideae* contains *inter alia* the genus *Cryptococcus* which has many features in common with *Rhodotorula*. The principal differences are that in *Cryptococcus* "manifest red to yellow pigments of carotenoid nature are lacking," (see pg. 371, ref. 13) and that species of *Cryptococcus* produce under appropriate conditions a starch-like compound, which is absent (with one exception) in *Rhodotorula*. We shall not consider here the taxonomic significance of the production of starch-like compounds, but shall report on the occurrence of carotenoid pigments in both genera. LODDER and KREGER-VAN RIJ relied mainly on visual observation of the slant cultures. This procedure is necessarily arbitrary so that some strains were included in *Cryptococcus* which showed "a slightly reddish or yellowish tinge in their streak culture" (see pg. 71 ref. 13). The literature contains no information on the pigments of yellowish yeasts which are either *Rhodotorula* or *Cryptococcus*, depending on the system of classification. We have, therefore, studied a number of strains belonging to

---

<sup>1)</sup> Present address at the University of California, Davis, California.

the above two genera, some of which were obtained from the C.B.S. culture collection and the remainder were isolated in our laboratory.

Both the nature and amount of carotenoid pigments in these yeasts were investigated. Moreover the observation of PHAFF *et al.* (15) that some strains of *R. glutinis* are red when grown at room temperature but appear yellowish at lower temperatures, was examined further by analysing the pigment composition at two temperatures of growth. For comparison we included a strain of *R. rubra* which remains red when grown at 5° C., and a strain of *R. penaeus*, a canary yellow yeast (15), which has a paler color at the lower temperature.

Yeast carotenoids have been reviewed recently by GOODWIN (6) and KARRER and JUCKER (7). Previous workers have shown that  $\beta$ -carotene and torulin are normally present in red yeasts, and have also observed an acidic pigment which may not invariably be present. LEDERER (11) discovered this pigment in *R. rubra*, which was confirmed by FINK and ZENGER (4). KARRER and RUTSCHMANN (8, 9, 10) also found the acidic pigment in *R. rubra*. They named it torularhodin and elucidated the main features and the constitution. However, BONNER *et al.* (1) could not demonstrate this pigment in any marked quantity in their strain of *R. rubra*. LODDER and KREGER-VAN RIJ (13) found the acidic pigment in several but not all red strains of *Rhodotorula* (not named). Torulin and torularhodin are responsible for the pink color in *Rhodotorula*. Lycopene and other minor components such as  $\gamma$ -carotene have at times been detected (1, 5, 12).

## EXPERIMENTAL METHODS AND MATERIALS.

### Culture material:

The organisms used during the study are described in Table I. In most cases the nomenclature adopted by LODDER and KREGER-VAN RIJ has been used. To avoid confusion, the older and more familiar synonyms of some strains are given in parentheses.

### Growth conditions:

The various cultures were first grown on several media to determine which would give the most intense pigmentation. After comparison of results on wort agar, potato agar dextrose, and yeast autolysate

TABLE I.

| Yeast                                                                                                       | Food<br>Tech-<br>nology<br>Collection<br>Number | Source                      | Approximate<br>Color<br>on Wort<br>Slant |
|-------------------------------------------------------------------------------------------------------------|-------------------------------------------------|-----------------------------|------------------------------------------|
| (1) <i>Rhodotorula rubra</i> (Demme) Lodder                                                                 | 50—323                                          | C.B.S. <sup>1)</sup>        | pink                                     |
| (2) <i>R. rubra</i> var. <i>longa</i> Lodder                                                                | 50—324                                          | C.B.S.                      | orange-red                               |
| (3) <i>R. rubra</i> var. <i>curvata</i> Lodder                                                              | 50—307                                          | C.B.S.                      | pale pink                                |
| (4) <i>R. glutinis</i> (Fres.) Harrison                                                                     | 48—23T                                          | F.T. <sup>2)</sup>          | pink                                     |
| (5) <i>R. glutinis</i> var. <i>infirmit-miniata</i><br>(Okunuki) Lodder                                     | 50—312                                          | from shrimp (15)<br>C.B.S.  | pink                                     |
| (6) <i>Cryptococcus laurentii</i> (Kuff.)<br>Skinner (formerly <i>R. aurea</i> (Saito)<br>Lodder, strain I) | 50—320                                          | C.B.S.<br>No. 40.5.1        | yellowish                                |
| (7) <i>Cr. laurentii</i> (Kuff.) Skinner (for-<br>merly <i>R. aurea</i> (Saito) Lodder,<br>strain II)       | 51—10                                           | C.B.S.<br>No. 40.5.2        | yellowish                                |
| (8) <i>R. flava</i> (Saito) Lodder                                                                          | 50—315                                          | C.B.S.                      | yellow                                   |
| (9) <i>R. peneaus</i> Phaff et al.                                                                          | 48—23A                                          | F.T.<br>from shrimp (15)    | canary yellow                            |
| (10) <i>Cryptococcus luteolus</i> (Saito) Skinner                                                           | 51—12                                           | C.B.S.<br>No. 24A.7.1       | weakly yellow                            |
| (11) <i>Cr. laurentii</i> (Kuff.) Skinner var.<br><i>flavescens</i> (Saito) Lodder et van<br>Rij            | 51—17                                           | C.B.S.<br>No. 24A.6.1       | cream                                    |
| (12) <i>Cr. laurentii</i> (Kufferath) Skinner                                                               | 51—277                                          | C.B.S.<br>from frozen beans | yellow                                   |
| (13) <i>Cr. laurentii</i> (Kufferath) Skinner                                                               | 51—278                                          | C.B.S.<br>from cotton bolls | yellow                                   |

<sup>1)</sup> Centraal Bureau voor Schimmelcultures, Yeast Division, Delft, Netherlands.<sup>2)</sup> Department of Food Technology, University of California, Berkeley, California.

agar with different concentrations of glucose, maltose or glycerol, it was decided that a medium consisting of 10 % (v/v) yeast autolysate (2), 2.5 % agar and 5 % glucose gave the greatest degree of pigmentation for most species. Red yeasts grown in a liquid medium of the same composition under aeration gave good growth but a low yield of carotenoids. Cotton-stoppered pint-sized flat bottles in which the agar was allowed to harden with the bottle lying horizontally were convenient containers for growth. Incubation was done in the dark with the bottle lying in the same position. Each bottle

was inoculated with 2 ml of yeast suspension taken from a young wort agar slant culture. Cultures were harvested by washing the yeast off the agar surface with water and glass beads. Extremely slimy organisms required a spatula. The washings were filtered through several layers of cheese cloth, thoroughly mixed, and aliquots removed for dry weight determinations.

### Pigment extraction:

Most of these colored yeasts are highly capsulated, which complicates extraction. With strain 48-23T of *R. glutinis*, autolysis at 32° C. for 2 days was highly successful, the pigment being completely extracted by stirring the autolysate with acetone. With the majority of strains, however, it did not give a complete or even a representative yield, and frequently no pigment could be extracted. The method of BONNER *et al.* (1), shaking with benzene and 20% KOH in methanol, also gave good results with some strains, but unrepresentative extractions in others. The failure to extract torularhodin efficiently from some red strains by this methods was particularly noted. We therefore retained a modified acid extraction procedure of MRAK, PHAFF and MACKINNEY (14) which gave results for the 48-23T strain identical with those obtained by autolysis. This is an important point in establishing the representative nature of the pigment extract by this method.

Concentrated HCl was added to the yeast suspension until the acid strength was 1.2 N. The mixture was then boiled for a short time. We found it possible to reduce the HCl concentration from 6 N as used by MRAK *et al.*, to 1.2 N and yet obtain a complete subsequent extraction. The boiling time was determined by the flocculation of the yeast, at which time the flask was cooled under running water. The yeast suspension was then centrifuged. In some cases it was necessary to add acetone up to 20 % to obtain good sedimentation. The carotenoid-free supernatant was discarded and the sediment was exhaustively extracted with anhydrous acetone, filtered through paper and then the filtrate was partitioned with petroleum ether.

The pigmented petroleum ether solution was then left overnight at 0° C. to precipitate colorless impurities. These may otherwise, interfere with the separations. At this stage, it is convenient to extract torularhodin, when present, from the petroleum ether so-

lution prior to further purification for chromatography. This pigment is readily extracted from petroleum ether with cold 0.1 N alkali. The difference in the absorption spectra of the petroleum ether solutions, before and after this extraction is a direct measure of the torularhodin content. It is the simplest and most direct measure of this pigment, since one avoids losses in subsequent saponification and in re-transfer back to petroleum ether.

In some cases, particularly those involving the yellow yeasts, appreciable quantities of lipid material may also be extracted. They interfere seriously with the subsequent chromatographic separation of the carotenoids. Much clearer zones are obtained in such instances, when the lipids are first removed by saponification. This was accomplished when necessary by shaking the petroleum ether extracts of the yellow yeasts and the corresponding extracts of some of the red yeasts (after removal of torularhodin) with 12 % methanolic KOH. The solutions of pigments in petroleum ether were then concentrated and dried with anhydrous  $\text{Na}_2\text{SO}_4$  prior to chromatography.

### Chromatographic Procedure:

The adsorbent used was MgO (Mallinckrodt) one part of which was mixed with 2 parts of  $\text{SiO}_2$  (Hy-Flo Supercel, Johns Manville). In the case of the red yeasts, a preliminary elution of weakly adsorbed components was effected on one column. This eluate was then concentrated and chromatographed again, and by this procedure three colorless bands were detected by their fluorescence under an ultraviolet lamp.

### Final Characterization:

The eluates of each zone were then examined in the Beckman DU spectrophotometer. The analyses for torulin are based on an extinction coefficient of  $E_{1\text{ cm.}}^{\text{mol.}} = 16 \times 10^4$  (11) and for torularhodin of  $E_{1\text{ cm.}}^{1\%} = 2.58 \times 10^3$  (9). Identification was made by position on the column, behavior in various solvents, absorption spectra, and co-chromatography with authentic specimens. In some cases the torularhodin fraction was crystallized and its melting point determined. In the case of *R. glutinis* 48-23T where extraction with acetone after autolysis gave complete recovery of the pigments, an absorption curve was plotted and compared with a curve of an acid extract of the same yeast. The curves had identical shapes indicating



that the acid extraction procedure gave a representative extract of all components present, and quantitatively there was no significant difference between the two methods.

### RESULTS.

The first five yeasts listed in Table I are without dispute to be classified as species of *Rhodotorula*. With the exception of Nr 5, they contain torularhodin. The combined torularhodin — torulin fraction accounts for 57 to 85 % of the total pigment (Table II) so that they are pink in color. The percentages reported for the different components are subject to considerable variation, as will be shown later. The pigment content, per unit dry weight is, however, in general agreement with the appearance on a wort slant. Thus *R. rubra* var. *curvata* has the lowest pigment content, and is the palest in color of these five organisms. Small quantities of torularhodin were crystallized from this variety of *R. rubra*. The crystals melted at 203° C. (corr.) and the absorption spectrum and partition behavior were determined (cf. 7, 9).

TABLE II.  
Pigments of a number of pink species of *Rhodotorula*.

| No.    | Name                                   | Percentages      |                   |         |                    | Total<br>Pigment<br>mg/100g<br>dry wt. |
|--------|----------------------------------------|------------------|-------------------|---------|--------------------|----------------------------------------|
|        |                                        | Beta<br>Carotene | Gamma<br>Carotene | Torulin | Torula-<br>rhodin  |                                        |
| 50-323 | <i>R. rubra</i>                        | 7.8              | 7.4               | 52.5    | 32.3               | 4.98                                   |
| 50-324 | <i>R. rubra</i><br>var. <i>longa</i>   | 10.3             | 7.2               | 17.5    | 65.0               | 17.24                                  |
| 50-307 | <i>R. rubra</i><br>var. <i>curvata</i> | 18.5             | 15.6              | 36.5    | 29.5               | 2.76                                   |
| 48-23T | <i>R. glutinis</i>                     | 33.4             | 9.6               | 14.5    | 42.5 <sup>1)</sup> | 6.85                                   |

<sup>1)</sup> In another strain, *R. glutinis* var. *infirmit-miniata* No. 50-312, torularhodin was absent.

The remaining yeasts listed in Table I vary from an off-white to canary yellow. The carotenoid content varied from 0.03 to 10 mg per 100 g dry yeast matter (Table III). The lowest, Nr 11, *Cr. laurentii* var. *flavescens* contained from 0.03 to 0.05 mg per 100 g. However, in a separate experiment, this organism was cultured in

Wickerham's nitrogen base plus 3 % glucose under shaking conditions and the carotenoid yield was at least 20 fold greater. A deep blue color was given with concentrated  $\text{H}_2\text{SO}_4$ . On chromatography, the same pattern of pigments was revealed as with the other members of *Cryptococcus*. In a previous paper (14) two of us reported that *Torulopsis luteola* (syn. *Cr. luteolus*) contained no carotenoid pigments, in tests based on a quantity of yeast equivalent to the growth in one petri dish. Here, however, the quantity of yeast used was more than ten times as great and the culture age (3 weeks) was appreciably longer.

TABLE III.

Summary of carotenoid data on 8 strains of yellow yeasts grown on yeast autolysate glucose agar.

| Yeast                                                    | Total mg/100 g dry wt. |
|----------------------------------------------------------|------------------------|
| (11) <i>Cr. laurentii</i> var. <i>flavescens</i> (51—17) | 0.03—0.05              |
| (10) <i>Cr. luteolus</i> (51—12)                         | 2.50                   |
| (7) <i>R. aurea</i> (51—10)                              | 9.0                    |
| (9) <i>R. peneaus</i> (48—23A)                           | 6.25                   |
| (8) <i>R. flava</i> (50—315)                             | 9.85                   |
| (6) <i>R. aurea</i> (50—320)                             | 6.98                   |
| (12) <i>Cr. laurentii</i> (51—277)                       | 4.55                   |
| (13) <i>Cr. laurentii</i> (51—278)                       | 5.5                    |

Whereas the first five members of *Rhodotorula* listed in Table I have a pattern based predominantly on torularhodin and/or torulin, the remaining yeasts, *Rhodotorula* or *Cryptococcus*, show a pattern mostly based on  $\beta$ -carotene which comprises 60 to 95 % of the total pigment content, with minor amounts of  $\gamma$ -carotene and lycopene. As in the previous case, the proportions can be markedly varied.

The petroleum ether extracts were fluorescent under ultraviolet radiation, and the chromatograms showed a zone of blue fluorescing material which could be readily eluted. In addition, two green fluorescing zones were observed. Those from *R. peneaus* and *Cr. laurentii*, 51-277 and 51-278 were isolated. Their absorption spectra did not correspond with phytofluene. The blue fluorescence was unaffected by iodine. The absorption spectrum of the blue fluorescing compound showed a maximum in petroleum ether at 340  $m\mu$  and points of inflection at 358 and 325  $m\mu$ . The eluates with green fluorescence had the typical 3-banded polyene spectrum with maxima at 408, 385 and 365  $m\mu$ .

## Effect of temperature on certain strains of *Rhodotorula* and *Cryptococcus*.

According to FROMAGEOT and TCHANG (5), the pigments from *R. sanniei* remain essentially the same qualitatively when the yeast is grown in the temperature range 14 to 28° C. PHAFF *et al.* (15) also found, by visual comparison, that most of their strains had the same color when grown at a high or a low temperature, but some behaved abnormally. They isolated two strains of *R. glutinis* which were salmon pink at 25° C. and dark yellow at 5° C. Furthermore their newly described species, *R. penaeus* was canary yellow at 25° C. and cream colored at 5° C.

It was found that the shade of color changed gradually when the incubation temperature was lowered, and the greatest contrast was obtained at the above mentioned temperatures. Comparisons were, therefore, made of the pigment composition at these two temperatures for *R. rubra* Nr 50-323 (which has the same color at 5° C. and at 25° C.), *R. glutinis* (48-23T), *R. penaeus* (48-23A), *R. aurea* (50-320) and *Cr. laurentii* (51-17). Little can be said of the last mentioned, since the total pigment concentration was less than 50  $\mu$ g per 100 g dry weight at either temperature under our conditions. The strain of *R. aurea* in this experiment was not highly pigmented, containing 0.25 mg per 100 g at 25°, 0.114 mg per 100 g at 5°. *R. penaeus* showed a marked drop in carotenoid synthesis from *ca.* 9 mg at 25° to *ca.* 1 mg  $\pm$  0.75 per 100 g dry yeast. Whereas at 25°, the  $\beta$ -carotene represented 93 % of the total, it fell to 66 % at the lower temperature. While less lycopene was synthesized at 5°, the proportion rose from 3 % at 25° to 13 % at 5° C.

The carotenoids of *R. rubra* were modified relatively little, though the absorption spectra for the extracts illustrate a decrease of *ca.* 16 % in the torularhodin and torulin fractions at 5°. The total carotenoid remained virtually unchanged at *ca.* 11 mg per 100 g.

In *R. glutinis* (48-23T), the trend shown by *R. rubra* is greatly intensified. A striking change occurs at the lower temperature as is shown in Figure 1. At 5° C.,  $\beta$ - and  $\gamma$ -carotene comprise 92-96 % of the total pigment, but only 43-47 % at 25° C. The remainder is comprised of torulin and torularhodin, which account for 4 to 8 % and 53 to 57 %, respectively. Thus the proportions of torulin and torularhodin drop to a level where they do not contribute any more to a visible pink. On the other hand, the total quantity of yellow

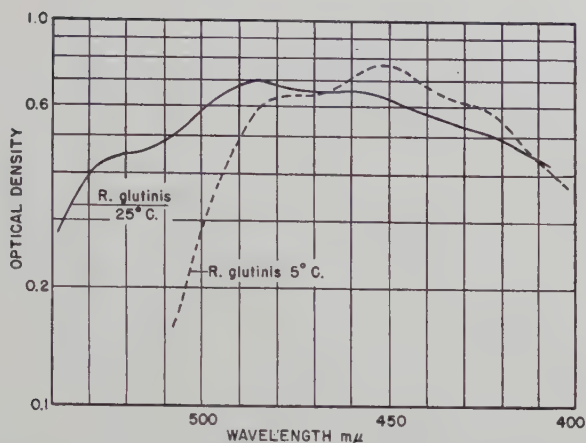


Fig. 1. Absorption spectrum of the carotenoid pigments of *R. glutinis* strain 48-23T grown for 3 weeks at 5° and 25° C.

carotenoids appears to increase appreciably at the low temperature, explaining the yellow color of the growth. Figure 2 shows the absorption curves of *R. rubra* and *R. peneaus* grown at 25° C. The similarity of the curves of the pink *R. glutinis* and *R. rubra* at 25° C. is quite apparent. However, the curve of the yellow *R. glutinis* grown at 5° C. and that of the yellow *R. peneaus* grown at 25° C. are also strikingly similar. These spectrophotometric results confirm the visual appearance on slant cultures.

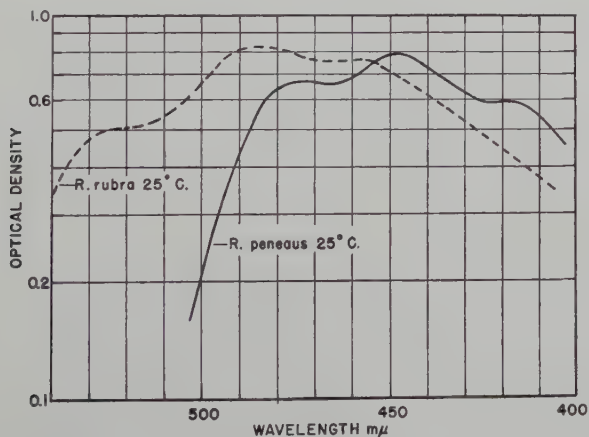


Fig. 2. Absorption spectrum of the carotenoid pigments of *R. rubra* and *R. peneaus* grown for 3 weeks at 25° C.

It should be added that in the series at 25° and 5°, cultures were incubated for a specific time, 3 weeks, not necessarily the time at which either optimal pigmentation or maximal differentials would be attainable.

#### DISCUSSION.

The data presented here show conclusively that the carotenoid content of a number of species of *Cryptococcus* may equal that of typical red species of *Rhodotorula*, even though the qualitative composition may be radically different. In *Rhodotorula* the proportions of torulin and torularhodin vary greatly and the torularhodin may even be absent in some strains. *R. aurea* was transferred to *Cryptococcus* by LODDER and KREGER-VAN RIJ (13) while *R. flava* was maintained in *Rhodotorula*. However, both species are shown here to have qualitatively and nearly quantitatively the same pigment composition ( $\beta$ -carotene as the major pigment). Among the species of *Cryptococcus* studied the carotenoid content varied from almost imperceptible amounts up to 10 mg per 100 g dry weight. These values represent averages for the total yeast crop harvested from agar surface. However, there may be appreciable variation in pigment content of a culture. For example, when grown on an agar surface the carotenoid content of the usually more brightly colored surface layer was found in some cases (particularly for *R. peneaus*) to be 10 times that of the bottom layer. Another complicating factor is the observation that when a trial run was made to grow *R. rubra* in shaking cultures, practically no pink pigments were formed, in spite of a good growth. On the other hand, when *Cr. laurentii* (51-17) was grown in shaking culture, the content of yellow carotenoids was greater than on a solid medium. The cultures should, where possible, be grown under homogeneous conditions, *i.e.* in shaking cultures. Unfortunately, carotenoid synthesis may then be slight. The effect of environmental conditions must, therefore, be evaluated in each case.

The effect of temperature of growth on carotenoid synthesis on solid medium was found to be quite variable for different species. One strain of *R. glutinis* described by PHAFF *et al.* (15) which exhibited strikingly different colors when grown at room temperature and 5° C. was almost devoid of torulin and torularhodin at the lower temperature although synthesis of the yellow carotenoids was greatly enhanced.



On the other hand, the  $\beta$ -carotene content of *R. peneaus* (major pigment of this yeast) was greatly reduced when grown at low temperatures, whereas the torulin, torularhodin,  $\beta$ - and  $\gamma$ -carotene contents of *R. rubra* were unaffected by the temperature. Due to different degrees and rates of autolysis, the dry weights of cells grown at different temperatures may vary and this will be reflected in the carotenoid per unit "apparent" dry weight.

Other factors which may cause variations in the carotenoid content of yeast are mutations (3, 1) and effect of various carbon sources in the medium of growth (16).

The variability in the carotenoid content and its qualitative composition in *Rhodotorula* and *Cryptococcus* suggests that a separation of the two genera, on the basis of manifest carotenoid is vague and arbitrary. If a separation is to be maintained between the two genera, it should be based on some other characteristic, possibly on the ability to produce the starch-like compound already mentioned. This has so far only been found in one strain of a red yeast, though it occurs regularly in the yellow yeasts except in *R. flava*. A taxonomic solution for the two genera in question will be considered in due course.

### S u m m a r y.

1. The carotenoid pigments of a number of species of *Rhodotorula* and *Cryptococcus* have been investigated.

2. In both genera, so-called yellow yeasts are found which contain  $\beta$ -carotene as the principal pigment.

3. The  $\beta$ -carotene content of species of *Cryptococcus* varies from nearly imperceptible amounts to 10 mg per 100 g dry yeast.

4.  $\beta$ -carotene is the main pigment of *R. peneaus*. The concentration of this pigment is greatly decreased when this yeast is grown at 5° C.

5. The type species of *R. rubra* was found to contain torularhodin, torulin, and  $\beta$ - and  $\gamma$ -carotene as the principal pigments. The composition of the pigments was quantitatively and qualitatively nearly the same, whether the yeast was cultured at 5° or at 25° C.

6. Certain strains of the red *R. glutinis* appear yellow when grown at 5° C. Under these conditions, the proportion of carotene increased appreciably, and torularhodin and torulin content fell correspondingly.

7. The taxonomic significance of carotenoid pigments for genus differentiation in the asporogenous yeasts is questioned.

#### Addendum.

A recent abstract (Bact. Proceedings 1953, p. 31) by PETERSON *et al.* shows that it is possible to extract the carotenoids of yeasts directly with cold acetone, without acid hydrolysis, provided the yeasts are grown in liquid yeast nitrogen base (Wickerham) plus glucose, on a rotary shaker. We have, at least qualitatively, confirmed the above finding with one red and one yellow yeast. We also found that the amount of pigment produced was much greater than in yeast autolysate glucose. This technique is very promising in growing cells for pigment studies under homogeneous conditions.

#### Bibliography.

1. BONNER, J., SANDOVAL, A., TANG, Y. W. and ZECHMEISTER, L. 1946. Arch. Biochem. **10**, 113.
2. BOUTHILET, R. J., NEILSON, N. E., MRAK, E. M. and PHAFF, H. J. 1949. J. Gen. Microbiol. **3**, 282.
3. CIFERRI, R. and REDAELLI, P. 1925. Atti 1st. Bot. Univ. Pavia, Serie III, Vol. II, p. 147—303.
4. FINK, H. and ZENGER, E. 1934. Wschr. Brauerei. **51**, 89.
5. FROMAGEOT, C. and TCHANG, J. L. 1938. Archiv f. Mikrobiol. **9**, 424.
6. GOODWIN, T. W. 1952. Botanical Rev. **18**, 291.
7. KARRER, P. and JUCKER, E. 1950. Carotenoids. Elsevier Publishing Co., Inc. New York. 384 pp.
8. KARRER, P. and RUTSCHMANN, J. 1943. Helv. Chim. Acta **26**, 2109.
9. KARRER, P. and RUTSCHMANN, J. 1945. Helv. Chim. Acta **28**, 796.
10. KARRER, P. and RUTSCHMANN, J. 1946. Helv. Chim. Acta **29**, 355.
11. LEDERER, E. 1933. Compt. rend. **197**, 1694.
12. LEDERER, E. 1938. Bull. Soc. Chim. Biol. **20**, 611.
13. LODDER, J. and KREGER-VAN RIJ, N. J. H. 1952. The yeasts. A taxonomic study. North Holland Publishing Co. Amsterdam. 713 pp.
14. MRAK, E. M., PHAFF, H. J. and MACKINNEY, G. 1949. J. Bact. **57**, 409.
15. PHAFF, H. J., MRAK, E. M. and WILLIAMS, O. B. 1952. Mycologia **44**, 431.
16. PHAFF, H. J. (Unpublished data).

(From the Bacteriological Laboratory of the St. Elisabeth Ziekenhuis,  
Tilburg, Holland).

## A SIMPLE METHOD FOR DETERMINING THE SENSITIVITY OF MICRO-ORGANISMS TO SERIAL DILUTIONS OF ANTIBIOTICS

by

**J. VAN DER VEEN and L. BOTS**

(Received August 22, 1953).

In routine sensitivity tests the use of solid media obviously facilitates the identification not only of the bacterial strains under test but also the recognition of any contaminants. The advantage of dilution methods, namely that they give direct quantitative results, is diminished, however, by the fact that these methods are rather complicated if the test has to include several antibiotics. Diffusion methods are usually simpler, and the sensitivity to different antibiotics may be measured in one and the same plate. However, they are less accurate than dilution methods. For this reason a dilution method employing solid media is preferred at this laboratory. In order to reduce the large amount of tubes or Petri dishes required for preparing dilutions of antibiotics, a simple modification has been devised for diluting the antibiotics in the cylindrical flat-bottomed cups of a specially constructed plastic dish which is described below.

### M a t e r i a l s.

The plastic dishes are made from "Akulon", a superpolyamide<sup>1)</sup>. The softening point of this superpolyamide lies between 215° and 220° C. Dishes with a thickness of 2 cm are sawed off from an "Akulon" bar with a diameter of 10 cm. Eight cups with a diameter of 18 mm and with a depth of 15 mm are bored along the periphery of the dish and one cup is made in the centre. The bottom of each cup is flat and polished. The dishes are covered with a lid of a Petri

---

<sup>1)</sup> „Akulon" bars are supplied by N.V. AKU, Arnhem, Holland.

dish. They may be sterilized by steam at  $121^{\circ}\text{C}$ . for 20 minutes.

Quantities of 0.05 ml of suitable dilutions of various antibiotics are pipetted into the cups along the periphery, the cup in the centre serving as control. An amount of 0.5 ml of melted blood agar is added to each cup and mixed with the antibiotics by rotating the dish over a flat surface. A thin layer of medium is formed in this way.

#### Performance of the sensitivity test.

The agar of each cup is inoculated with one drop (0.02 ml) containing about 10 thousand bacteria in saline. After incubation at

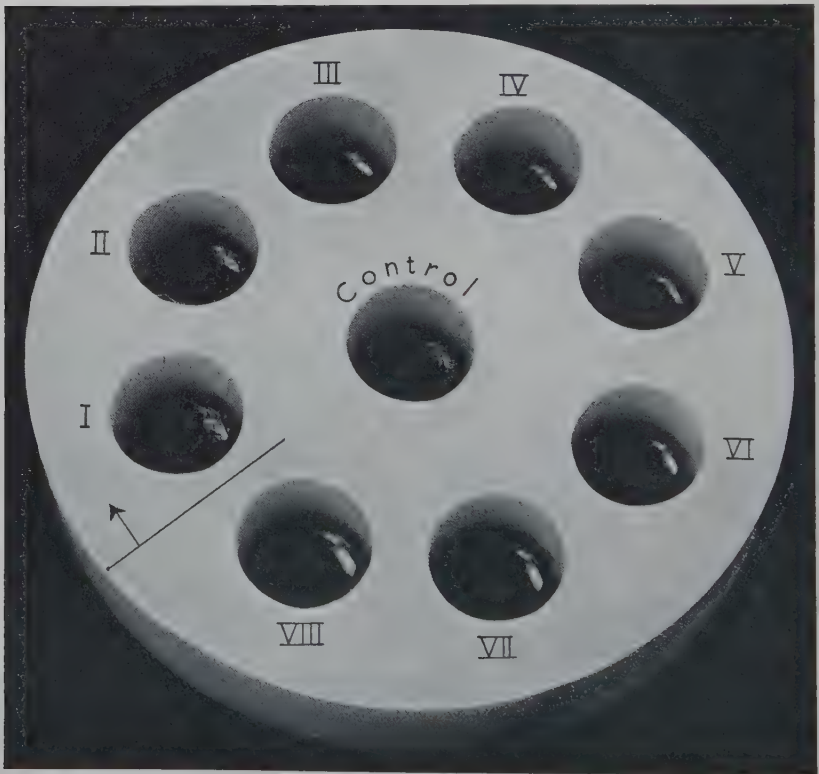


Fig. 1. Sensitivity test of a *Staphylococcus* against serial dilutions of penicillin; bacterial growth is visible on agar containing 0.025 and 0.05 U penicillin per ml (cups No. I and II), but is completely inhibited by 0.1 U penicillin per ml (cup No. III).

37° C. for 20 hours the colonies are not confluent, but uniformly distributed over the surface of the agar. The various bacterial species are easily identified and haemolysis may be seen with transmitted light. All factors which may influence the results of the tests are kept constant, e.g. the size and age of the inoculum, the medium and the time of incubation. A record is made of the lowest concentration of antibiotic at which macroscopic growth of each organism is completely inhibited (Fig. 1).

### Control of the technique.

The accuracy of the technique has been tested by performing a series of 8 sensitivity tests with a *Staphylococcus* against sulfathiazol and against each of the following antibiotics: penicillin, streptomycin and chloramphenicol, and with a strain of *Escherichia coli* isolated at this laboratory, against aureomycin and terramycin. Two-fold serial dilutions of the antibiotics were made. Identical results were obtained in all tests except that sometimes small differences in the size and in the quantity of the colonies were seen in the highest dilution with visible growth.

Sensitivity tests with a standard strain of known sensitivity are carried out each time after the dishes are newly prepared. The *Staphylococcus* strain is employed as test strain for sulfathiazol, penicillin, streptomycin and chloramphenicol. A strain of *Escherichia coli* is employed as test strain for aureomycin and terramycin. Each series of dishes with the same antibiotic is checked by inoculating one dish with the standard strain. A significant variation (more than a twofold dilution) in the endpoint of growth of the standard strain indicates that the dishes are improperly prepared. In these cases the other dishes with the same antibiotic are discarded. Up till now we have not found any significant variation in the results of sensitivity tests of the standard strain against sulfathiazol, penicillin and chloramphenicol. However, a few times the lowest concentrations of streptomycin, aureomycin and terramycin, at which growth of the standard strain was inhibited, showed a variation in one or more dilutions.

The keeping qualities of the various diluted antibiotics incorporated in the blood agar medium and kept at 4° C. were checked by continual control with the *Staphylococcus* strain or with a strain of *Escherichia coli*. It appeared that sulfathiazol, penicillin, strepto-



mycin and chloramphenicol were stable for at least one week. However, aureomycin deteriorated after one day and terramycin after two days. Therefore, dishes with aureomycin and terramycin have to be made just before use; dishes with the other substances mentioned above may be prepared once every week.

### S u m m a r y.

At this laboratory the routine sensitivity tests of micro-organisms against sulfathiazol and antibiotics have been performed in plastic dishes for half a year. The dishes are cleaned and sterilized in the same way as bacteriological glassware. The use of these dishes offers some advantages over other conventional dilution methods employing tubes or Petri dishes with solid media: the sensitivity to serial dilutions of one antibiotic may be measured in one and the same plate, and in this way a considerable amount of glassware and of space in the incubator may be saved; the readings are easily done and distinct. It has been demonstrated that sensitivity tests with two standard strains against sulfathiazol and various antibiotics may be reproduced with the same endpoints.

---

(Central Institute for Nutrition Research T.N.O., Utrecht, The Netherlands).

## A GELATIN LIQUEFACTION TEST FOR THE SCREENING OF COMPOUNDS USED OR PROPOSED AS INHIBITORS OF BACTERIAL PROTEOLYTIC DETERIORATION IN FOODS

by

**D. A. A. MOSSEL and A. S. DE BRUIN**

(Received June 19, 1953).

For the systematic evaluation of antimicrobial compounds, proposed as food preservatives, it was felt necessary to use tests modelled on the types of deterioration occurring in practice (10). In the course of this investigation satisfactory tests have been developed for fungal spoilage and for fermentation by yeasts or bacteria (11).

The purpose of this paper is to report the development of a simple test for bacterial proteolysis, such as may occur during the spoilage of meat, fish, poultry and eggs.

### PRINCIPLE.

The test is modelled on the conditions at the surface of a spoiling proteinaceous food, as closely as is practicable for routine operations.

The food, with its moist surface, is represented by a nutrient gelatin slant. The spoilage flora is represented by an inoculum of psychrophilic Gram-negative rods, this being the type of organism responsible for slime and putrefaction in proteinaceous foods stored under cool conditions (6, 2, 3; 14, 1, 4). Surface inoculation is preferred to a poured-plate technique, since in practice the proteolysis occurs mostly at the surface, and it has been shown that oxygen tension may influence the rate of liquefaction of gelatin (9). The temperature is maintained at an appropriate level: about 25° C.

The breakdown of the gelatin is followed simply, by a modification of FERMI'S technique (5), i.e. by withdrawing and measuring the volume of the fraction liquefied.

## EXPERIMENTAL PROCEDURE.

As i n o c u l u m a Gram-negative rod — isolated from slightly slimy, refrigerated fish by subculturing at 5° C. — is employed, since such strains are frequent among the micro-organisms generally found on proteinaceous foods, spoiling at lower temperatures. The strain used forms acid but no gas from dextrose, laevulose, galactose, l-arabinose and d-xylose but no acid from sucrose, maltose, lactose, raffinose, glycerol and mannitol; gelatin is liquefied and tributyrin is saponified, but nitrite cannot be detected after 48 h incubation in 0.1% NaNO<sub>3</sub>-broth and neither is indole formed in tryptone medium. The organism is very actively motile in Tittsler's medium (16). Electron-microscopical examination of the strain revealed that it possessed terminal flagellae. Growth occurs within one week at 5, 24, 30, 32 and 37° C., but not at 45 and 55° C. These data suggest that the isolate is a *Pseudomonas* species.

The medium used in general is 12% nutrient gelatin (according to the Difco formula; pH<sub>0</sub> = 6.7), but for experiments especially designed to represent fish or egg spoilage, the 0.3% beef extract of this formula is replaced by aliquots of fish solubles and whole egg powder respectively.

Dissolving, sterilization and cooling of the media are standardized in order to eliminate differences in rheological properties. Dissolving of the dehydrated medium is carried out in lots of 600 ml. For this purpose a water bath at 55° C. is used; dissolving requires 15 min. The medium is sterilized for 15 min. at 121° C. in a pressure cooker (12). Beforehand the filled cooker is exhausted for 5 min. After sterilization the medium is cooled in water under shaking; it takes about 5 minutes to reach 50° C.

After cooling to 50° C.,  $6 \pm 0.05$  ml of the molten medium are poured in  $1.5 \times 15$  cm culture tubes and allowed to solidify at  $\leq 15^\circ$  C. in a slightly inclined position.

The slants obtained are i n o c u l a t e d by evenly spreading over the surface a layer of a 24 h/25° C. subculture on agar, derived from a fresh culture of the strain on nutrient gelatin. For this purpose a nichrome microspatula of  $3\frac{1}{2} \times 2\frac{1}{2}$  mm, prepared by flattening part of an inoculating needle, is used. In inoculating as much culture is used as covers the microspatula completely. Counts on nutrient agar at 24° C. revealed that the inoculation effected in this way corresponds to the order of  $10^9$  cells/tube, i.e.  $10^8$  cells/cm<sup>2</sup>.

After 72 h incubation at  $24 \pm 1^\circ$  C. the liquid formed is withdrawn in a 1 ml pipette (in several portions if necessary) and the total volume read to the nearest 0.01 ml.

## RESULTS AND DISCUSSION OF THEIR ACCURACY.

### Reproducibility.

In an initial series of experiments the reproducibility of the technique was investigated. Four technicians made independent observations on 1—4 series of six replicate tubes. The volumes of liquid obtained after 72 h incubation at  $24^\circ$  C. are shown in Table I.

TABLE I.  
Reproducibility of gelatin liquefaction test.

| Initials of technician | Volume (ml) of liquefied gelatin formed from 6 ml solid medium |      |      |                   |
|------------------------|----------------------------------------------------------------|------|------|-------------------|
|                        | Data                                                           | Mean | S.D. | C <sub>var.</sub> |
| A.S.B.                 | 4.83-4.60-4.77                                                 |      |      |                   |
|                        | 4.63-5.07-4.89                                                 | 4.80 | 0.17 | 3.6               |
|                        | 5.45-5.54-5.65                                                 |      |      |                   |
|                        | 5.39-5.56-5.58                                                 | 5.53 | 0.10 | 1.8               |
|                        | 5.37-5.33-5.32                                                 |      |      |                   |
|                        | 5.42-5.55-5.48                                                 | 5.41 | 0.09 | 1.7               |
| J.G.M.                 | 3.20-3.11-3.06                                                 |      |      |                   |
|                        | 3.07-3.19-3.01                                                 | 3.11 | 0.08 | 2.4               |
|                        | 4.36-4.82-4.56                                                 |      |      |                   |
|                        | 4.55-4.61-4.44                                                 | 4.56 | 0.16 | 3.5               |
|                        | 4.84-4.98-4.90                                                 |      |      |                   |
|                        | 4.58-5.05                                                      | 4.87 | 0.18 | 3.7               |
| H.P.                   | 4.39-4.71-4.55                                                 |      |      |                   |
|                        | 4.86-4.91                                                      | 4.68 | 0.22 | 4.8               |
|                        | 4.95-5.25-5.31                                                 |      |      |                   |
|                        | 4.89-5.03-5.26                                                 | 5.12 | 0.18 | 3.5               |
|                        | 5.34-5.43-5.37                                                 |      |      |                   |
|                        | 5.49-5.44-5.42                                                 | 5.42 | 0.05 | 1.0               |
| J.F.V.                 | 4.81-4.99-4.78                                                 |      |      |                   |
|                        | 4.98-4.78-4.72                                                 | 4.84 | 0.11 | 2.3               |
|                        | 3.09-3.39-3.27                                                 |      |      |                   |
|                        | 3.15-3.14-3.13                                                 | 3.20 | 0.11 | 3.5               |

The coefficients of variation of the measurements may apparently vary from 1.0—4.8, which is an acceptable accuracy for quantitative microbiological work.

The degree of liquefaction after 3 days at 24° C. varied from 52 to 92%, which is a fair value for this type of investigation (8). The differences in the degree of liquefaction could not be correlated with any variable other than intrinsic variation in the liquefying capacity of the strain. As will be shown later, these variations do not significantly influence the outcome of the evaluation of inhibitors, however.

### Testing of preservatives.

A number of preservatives in current use, and some recently developed antimicrobial compounds, were independently tested by two technicians. For this purpose the preservatives were weighed into sterilized Erlenmeyer flasks and then dissolved in the required volume of sterilized nutrient gelatin. No special measures were taken to sterilize thermolabile agents, since it was felt that the few germs introduced into the medium in this way would be overgrown by the massive inoculum of the *Pseudomonas* strain. The results obtained in practice confirmed this idea.

It is well known that the antimicrobial action of many preservatives depends on the initial pH of the medium. This holds true for the weak acids, such as benzoic and propionic acid (13), for nitrites (15) and of course for hexamine, which acts as a donor of formaldehyde. Therefore these compounds were tested both at the natural pH<sub>0</sub> of the gelatin medium ( $6.7 \pm 0.1$ ) and at pH<sub>0</sub> =  $5.5 \pm 0.1$ , which is a value that can be obtained in meat as a result of post-mortem formation of lactic acid from glycogen (7).

Before this could be done, it was necessary, however, to study the influence of pH<sub>0</sub> on gelatin liquefaction by the strain used.

The range pH<sub>0</sub> = 6.7 — 4.5 was therefore included in a series of experiments. The results obtained (Table II) at any pH<sub>0</sub> were expressed as relative liquefaction, in this case defined as:

$$L_R = \frac{\text{vol. \% liquefaction at pH}_0 = i}{\text{vol. \% liquefaction at pH}_0 = 6.7 \pm 0.1} \times 100$$

These data reveal that, although the present strain develops most gelatin liquefaction at pH<sub>0</sub>  $\approx$  6.7, marked inhibition (compared with



TABLE II.

Influence of initial pH on the liquefaction of gelatin in the technique under investigation.

| pH <sub>0</sub> | L <sub>R</sub> (average of 2 sets of 6 measurements) |         |
|-----------------|------------------------------------------------------|---------|
|                 | Extreme values                                       | Average |
| 6.7             | —                                                    | 100     |
| 6.0             | 99—93                                                | 96      |
| 5.5             | 89—88                                                | 89      |
| 5.0             | 54—64                                                | 59      |
| 4.8             | 24—26                                                | 25      |
| 4.5             | 0—0                                                  | 0       |

L<sub>R</sub>-data observed at pH<sub>0</sub> = 6.7) occurs only at a pH<sub>0</sub> < 5.5. The initial pH of about 5.5 could therefore be safely used.

The results obtained with the various preservatives are summarized in Table III. The activity of every agent has been expressed as AD<sub>50</sub> = concentration of the compound, which effects 50% re-

TABLE III.

Antimicrobial potency of various preservatives expressed as AD<sub>50</sub> = concentration corresponding with L<sub>R</sub> ∼ 50%.

| Preservative                                                                       | AD <sub>50</sub> (%) |
|------------------------------------------------------------------------------------|----------------------|
| NaCl                                                                               | 1.0                  |
| NaNO <sub>3</sub>                                                                  | 1.0                  |
| NaNO <sub>2</sub> , pH <sub>0</sub> = 5.5                                          | 0.05                 |
| SO <sub>2</sub> (added as Na <sub>2</sub> SO <sub>3</sub> ), pH <sub>0</sub> = 5.5 | 0.03                 |
| Boric acid                                                                         | 0.10                 |
| Benzoic acid, pH <sub>0</sub> = 5.5                                                | 0.20                 |
| Propionic acid, pH <sub>0</sub> = 5.5                                              | 0.10                 |
| Hexamine                                                                           | > 2.0                |
| Hexamine, pH <sub>0</sub> = 5.5                                                    | 0.20                 |
| Sodium methylparahydroxybenzoate                                                   | 0.05                 |
| Cetyltrimethylammoniumbromide                                                      | 0.10                 |
| Zephiran                                                                           | 0.01                 |
| Sodium dehydroacetate                                                              | 1.0                  |
| Same, pH <sub>0</sub> = 5.5                                                        | 0.2                  |
| Butyl gallate                                                                      | > 0.1                |
| Ethyl monobromoacetate                                                             | 25 mg Br/kg          |

duction in relative liquefaction, i.e.

$$\frac{\text{vol. \% liquefaction in preserved system}}{\text{vol. \% liquefaction in blank}} \times 100. \quad \text{Each value has}$$

been derived from the results obtained in 6 tubes by two technicians, working independently. The accuracy of the individual data closely followed the pattern given in Table I.

#### Influence of external factors.

It was imperative to study the effect of the following external factors:

- (a) intrinsic variations in gelatin liquefaction in the strain used;
- (b) any differences inherent to various batches of gelatin.

For purpose (a) the measurements reported in Table III were duplicated in cases, where low blank liquefaction data were obtained (cf. Table I). Even with differences in absolute liquefaction amounting to about 2.5 ml, the  $L_R$ - and consequently  $AD_{50}$ -values did not vary by more than the order 10% absolute, i.e. the spreading obtained in all experiments.

For purpose (b) the measurements reported in Table III were duplicated with different lots of nutrient gelatin. Here again, the influence exerted by the substratum was not surpassing the normal degree of variation in the results obtained.

#### DISCUSSION OF THE RESULTS OBTAINED.

The  $AD_{50}$ -values in general are in agreement with the recommendations for the use of the agents, based on actual practice, *viz.*, 10–50 mg Br/kg of bromoacetic acid derivatives, 0.01% for the best quaternary ammonium compounds, 0.02–0.05% for nitrites (15) and  $SO_2$  and the order 0.1% for the other compounds at suitable pH-values.

The most striking result is no doubt the lack of bacteriostatic efficiency of the strongly fungi- and mycostatic agent dehydroacetic acid (17, 11) at normal pH-values. The first indications in this respect were obtained by Wolf (17) and confirmed by the present authors (11). Meanwhile the same results have been obtained in other laboratories (13).

The close correlation between the outcome of the present test and the results obtained in practice, suggests that the test may be a useful tool in the screening of antibacterial agents in general.

### Summary.

1. A gelatin liquefaction test, useful in screening of inhibitors of bacterial proteolytic spoilage in foods, is described.

In this test gelatin slants are used as a substratum and a *Pseudomonas* strain, isolated from the slime formed on fish, stored at 5°C., is used as an inoculum. The preparation of the medium and the density of the inoculum are standardized.

The coefficients of variation of the absolute liquefaction data obtained by four technicians, when using the same batch of gelatin and inoculum, roughly vary from 1—5. The spread in the relative liquefaction data ( $L_R$ ), obtained for various preservatives, amounts to 10% absolute and is not significantly influenced by the use of different batches of gelatin or by differences in intensity of gelatin liquefaction in the strain.

2. Under the circumstances of the test, reduction in  $L_R$ -values of the order of 50% was obtained with: 0.05% sodium nitrite at  $pH_0 = 5.5$ , 1% sodium chloride, 0.1% propionic acid at  $pH_0 = 5.5$ , 0.1% boric acid, 0.2% benzoic acid at  $pH_0 = 5.5$ , 0.2% hexamine at  $pH_0 = 5.5$ , 0.05% sodium methylparahydroxybenzoate, 0.01% zephiran, 0.1% cetyltrimethylammoniumbromide and 1.0% sodium dehydroacetate, and ethyl monobromoacetate in a concentration corresponding to 25 mg Br/kg.

### References.

1. ASCHEHOUG, V. and VESTERHUS, R. 1947. Food Res. **12**, 55.
2. AYRES, J. C., OGILVY, W. S. and STEWART, G. F. 1950. Food Technol. **4**, 199.
3. AYRES, J. C. 1951. Iowa State College J. Sci. **26**, 31.
4. CASTELL, C. H. and ANDERSON, G. W. 1948. J. Fish. Research Board Canada **7**, 370.
5. FERMI, C. 1891. Arch. Hygiene **12**, 240.
6. HAINES, R. B. 1933. J. Hygiene **33**, 175.
7. INGRAM, M. 1948. Ann. Inst. Pasteur **75**, 139.
8. LEVINE, M. and CARPENTER, D. C. 1923. J. Bact. **8**, 297.
9. MERRILL, A. T. and CLARK, W. M. 1928. J. Bact. **15**, 267.
10. MOSSEL, D. A. A. and WESTERDIJK, J. 1949. Antonie van Leeuwenhoek **15**, 190.
11. MOSSEL, D. A. A. and DE BRUIN, A. S. 1950. Antonie van Leeuwenhoek **16**, 393.
12. MOSSEL, D. A. A. 1951. Chem. Weekbl. **47**, 611.

13. VON SCHELHORN, M. 1953. Food Technol. **7**, 97.
  14. SCHÖNBERG, F. und DEBELIC, S. 1933. Berl. tierärzt. Wschr. **49**, 396.
  15. TARR, H. L. A. and DEAS, C. P. 1948. J. Fish. Research Board Canada **7**, 221.
  16. TITSLER, R. P. and SANDHOLZER, L. A. 1936. J. Bact. **31**, 575.
  17. WOLF, P. A. 1950. Food Technol. **4**, 294.
-

(Laboratory of Microbiology, Technological University, Delft, Holland).

## STUDIES ON TRUE DISSIMILATORY NITRATE REDUCTION

### II. THE MECHANISM OF DENITRIFICATION<sup>1)</sup>

by

A. J. KLUYVER and W. VERHOEVEN

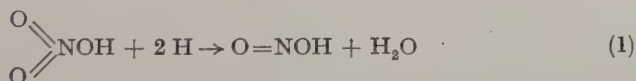
(Received January 27, 1954).

#### INTRODUCTION.

In two preceding publications (VERHOEVEN 1952, KLUYVER 1953) a scheme has been given for the chemical conversions to which nitrate is subject when it acts as a hydrogen acceptor in true dissimilatory nitrate reduction. In this publication we wish to consider more especially the problem of the formation of molecular nitrogen in the so-called denitrification process. There is some reason to return to this question, since this problem has recently also received the attention of two groups of American investigators (SACKS and BARKER 1952; ALLEN and VAN NIEL 1952). Their results could only be superficially considered in our last publication, and since then we have been able to carry out some further investigations in connection with their work.

It is clear that in denitrification nitrate acts as an acceptor either of hydrogen derived from some organic substrate, or of molecular hydrogen.

There seems to be general agreement between the various workers that the first step in nitrate reduction leads to the formation of nitrite, according to the equation:

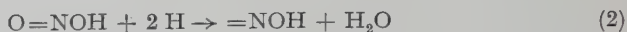


Taking this into account, it is only logical to postulate that the next step in the hydrogenation will be the removal of the second

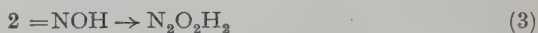
<sup>1)</sup> Part I: *Antonie van Leeuwenhoek* **20**, 93, 1954.



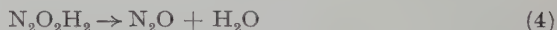
double bound oxygen atom from the nitrogen atom according to the equation:



The initial formation of the non-existent free radical  $=\text{NOH}$  is, of course, purely hypothetical. It should, however, be pointed out that the postulation of its occurrence has some unmistakable advantages. In the first place it makes acceptable the formation of  $\text{H}_2\text{NOH}$ , if further hydrogenation takes place as for instance in assimilatory nitrate reduction. Secondly in the case of denitrification the extreme reactivity of the free radical offers a ready explanation for the occurrence of reduction products containing two nitrogen atoms, like nitrous oxide and molecular nitrogen. A simple dimerization of the free radical to a compound of the elementary composition  $\text{N}_2\text{O}_2\text{H}_2$  seems to be a quite acceptable possibility:



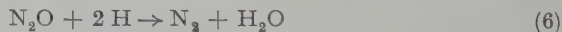
Leaving the nature of the compound  $\text{N}_2\text{O}_2\text{H}_2$  out of consideration for the moment, it is clear that it could well be converted into nitrous oxide by splitting off a molecule of water:



On the other hand a continued hydrogenation of the postulated intermediate product might well yield molecular nitrogen:



In the meantime the possibility cannot be excluded that the final hydrogenation step only takes place after the water has already been split off from the intermediate compound (reaction 4), which would mean that nitrous oxide is the precursor of molecular nitrogen:



The question arises whether it is possible to give experimental support to the foregoing theoretical considerations. A direct proof for the occurrence of the so reactive free radical  $=\text{NOH}$  as an intermediate does not offer much prospect. However, it seems worthwhile to attempt an identification of its dimer:  $\text{N}_2\text{O}_2\text{H}_2$ .

As such hyponitrous acid has already been suggested by several of the earlier authors, but quite recently ALLEN and VAN NIEL (1952) have postulated that the compound known as nitramide

might well be the intermediate in question, and they have offered some experimental evidence in favour of this view.

One of the aims of this study has been to reinvestigate the suitability of both hyponitrous acid and nitramide as intermediates in denitrification; this question will be dealt with in Part I.

Secondly we wished to collect additional experimental evidence regarding the question in how far molecular nitrogen always originates by a direct hydrogenation of the postulated intermediate, or that nitrous oxide is a regular or facultative precursor. The facts that SACKS and BARKER (1952) definitely reject nitrous oxide as an intermediate in nitrogen formation, whilst ALLEN and VAN NIEL (1952) are inclined to minimize the importance of nitrous oxide as such, seemed to justify a further study of this point. We shall consider this question in Part II of this publication.

## PART I.

### THE ACCEPTABILITY OF HYPONITRITE AND NITRAMIDE AS INTERMEDIATES IN DENITRIFICATION

#### *a. Experiments with hyponitrite.*

A sample of sodium hyponitrite was kindly put at our disposal by Professor G. MEYER, head of the Department of Inorganic Chemistry of the Technological University. We feel greatly indebted to Prof. MEYER for this gift.

Since the preparation which had been made by the reduction of nitrite with sodium amalgam still gave a rather strongly positive nitrite reaction according to Griess-Romeyn it was subjected to a further purification. About 9 gram of the hyponitrite was dissolved in 50 ml bidistilled water which had been cooled with ice. To the solution 150 ml ice-cold ethanol was added, which led to the precipitation of the hyponitrite, the nitrite remaining dissolved. The precipitate was collected on a Buchner funnel and washed with 50 ml cold ethanol. This procedure was repeated twice. After this treatment the Griess-Romeyn reaction of the preparation was only very faintly positive. On addition of silver ions to a solution of the preparation a characteristic bright yellow, dense precipitate was formed. In accordance with the experience of ALLEN and VAN NIEL the quantitative analysis of the hyponitrite proved to be rather difficult. A gas analysis in which the preparation was decomposed with acid under formation of nitrous oxide did not yield a higher value than

75 % hyponitrite, on the other hand titration with acid on methyl orange did give values surpassing 100 %.

This preparation has been used in the following experiments in which either *Pseudomonas aeruginosa* strain 2 or *Micrococcus denitrificans* strain BEIJERINCK have been applied as denitrifying organisms.

The bacteria were always cultivated under anaerobic conditions at 30° C. on peptone agar to which 1 %  $\text{KNO}_3$  and 0.5 % glycerol had been added. The pH of the medium was initially adjusted to 7.2.

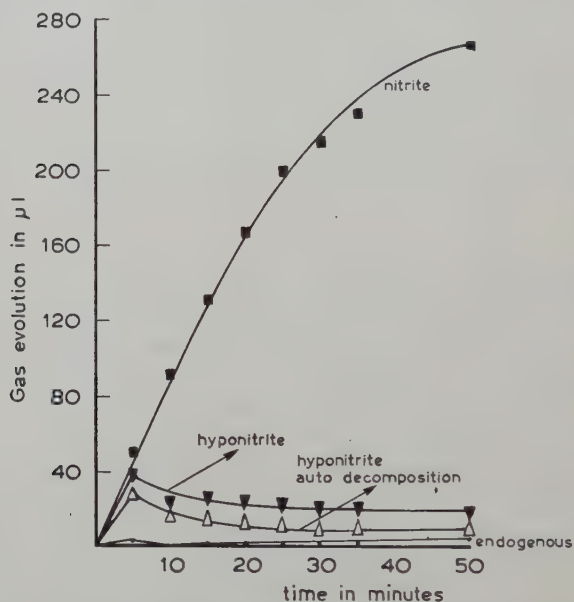


Fig. 1. Gas evolution by *Micrococcus denitrificans* from nitrite and hyponitrite in a nitrogen atmosphere. Hydrogen donator: glucose. Phosphate buffer: pH 7.2. Bacteria grown under anaerobic conditions on: peptone agar, 1%  $\text{KNO}_3$  and 0.5 % glycerol.

From 48 hours old cultures of *M. denitrificans*, or 20-24 hours old cultures of *Ps. aeruginosa* the bacteria were collected in phosphate buffer (1/15 mol, pH = 7.2), centrifuged, and once more washed with the same buffer.

In the manometric experiments 1.5 ml of a bacterial suspension in the said buffer were brought into the main vessel of a Warburg apparatus, after which 0.2 ml of a 1 % glucose solution was added. In the centre cup 0.2 ml 20 % KOH with a slip of filter paper took care of carbon dioxide absorption. In order to prevent an untimely

decomposition of the hyponitrite a small weight of the preparation — from 2 to 3 mg — was put in the side-arm of the vessel in the dry state. After gas equilibrium had been attained in the vessel the hyponitrite was dissolved in the bacterial suspension by tilting the apparatus.

In each series were also included: a vessel in which the hypo-

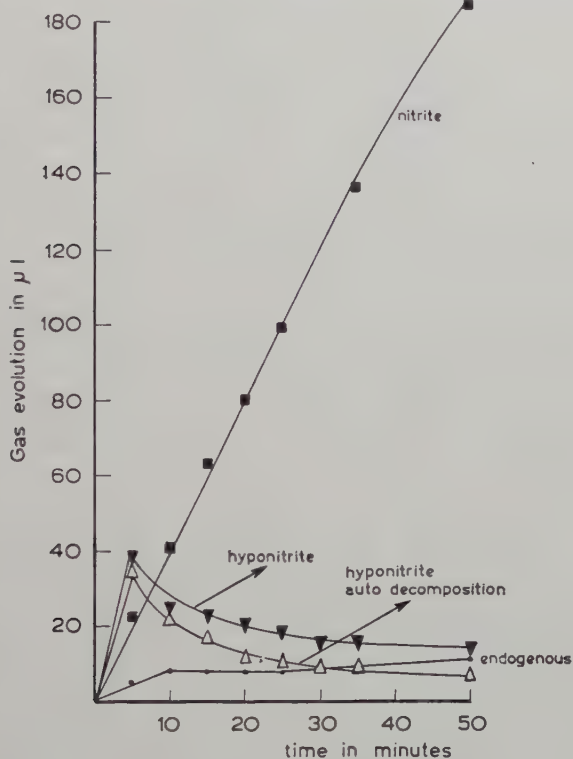


Fig. 2. Gas evolution by *Pseudomonas aeruginosa* from nitrite and hyponitrite in a nitrogen atmosphere. Hydrogen donor: glucose. Phosphate buffer: pH 7.2. Bacteria grown under anaerobic conditions on: peptone agar, 1 %  $\text{KNO}_3$  and 0.5 % glycerol.

nitrite had been replaced by nitrite in order to check the activity of the bacteria, a blank to which no acceptor had been added, and finally a vessel containing hyponitrite and buffer solution but without bacteria. This was done to investigate a possible auto-decomposition of the hyponitrite. In all vessels the air was replaced by nitrogen freed from traces of oxygen by passing it over a red-heated copper spiral.

Fig. 1 and Fig. 2 give graphs of the amount of gas evolved in two series of experiments made with *M. denitrificans* and *Ps. aeruginosa* respectively.

The results obtained show conclusively that the hyponitrite, in contrast to what holds for nitrite, is unable to give rise to nitrogen evolution in the presence of the denitrifying bacteria and a suitable hydrogen donor.

It is also evident that at least at pH = 7.2 no spontaneous decomposition of hyponitrite occurs, although in all experiments in the first 5 minutes a small quantity of gas is evolved which remarkably gradually disappears again. No explanation can be offered for this phenomenon which does not affect the main conclusion.

It should be added that in some special experiments we convinced ourselves that the hyponitrite did not have any toxic effect on the bacteria: in experiments in which a mixture of nitrite and hyponitrite was added to the bacterial suspension the gas evolution proceeded in exactly the same way as in the experiments in which nitrite was added exclusively.

#### b. Experiments with nitramide.

The encouraging results obtained by ALLEN and VAN NIEL (1952) regarding the suitability of nitramide as a hydrogen acceptor in denitrification induced us to investigate also this compound.

Professor MEYER again was so kind to supervise the preparation of this compound of which a generous sample was made available to us. In the preparation the prescription given by MARLIES, LA MER and GREENSPAN (1939) was strictly adhered to. The characteristics of the product obtained answered in all respects those of nitramide as described by the authors cited. It was a crystalline product appearing as pure white shiny leaflets which melted at 75.5° C. (corr.) with simultaneous decomposition. VON BRAUN (1907) reports melting points between 72 and 75° C., also with decomposition. It was found that on addition of 0.1 n KOH a strong gas evolution occurred. With the aid of the van Slijke apparatus it was established that the total volume of gas produced was in almost perfect agreement with the amount which might be expected on the assumption that one molecule of nitramide on decomposition yields one molecule nitrous oxide, as appears from the following figures:

|                                                       |                    |
|-------------------------------------------------------|--------------------|
| Calculated: 7 mg $\text{NH}_2\text{NO}_2 \rightarrow$ | 0.113 millimol gas |
| Found:                                                | 0.114 millimol gas |

Moreover, a combustion experiment in a mixture with hydrogen gas, also performed in the van Slijke apparatus, left no doubt that the gas ob-



tained was, indeed, pure nitrous oxide, since free oxygen can be considered to be absent in this case.

Preliminary experiments in the Warburg apparatus in which the nitramide was brought in the dry state in the side-arm — as previously described for hyponitrite — led to unexpected results. Immediately after tilting the apparatus in order to dissolve the nitramide in the bacterial suspension an extremely vigorous gas evolution occurred. The pressure in the manometer rose so quickly that no measurements were possible. This behaviour could hardly be due to the metabolic activity of the bacteria, a spontaneous decomposition seemed to be much more probable. This was confirmed by experiments in which the bacteria were omitted, and in which the nitramide was simply brought into contact with the phosphate buffer pH = 7.2. Here too an immediate vigorous gas evolution occurred.

A study of the literature showed that the instability of nitramide in aqueous solution has also been established in the earlier chemical studies on this compound. BRÖNSTED (1924) has studied the decomposition in detail, and has found that the rate of decomposition is accelerated by an alkaline reaction. He remarks that the decomposition in diluted hydrochloric acid at 15° C. is slow enough for measurement.

Since our results differed so markedly from the observations reported by ALLEN and VAN NIEL, we have thought it desirable to study the behaviour of our nitramide preparation in buffers in various pH ranges. We tested buffers with pH 5.6, 6.2, 6.6, 6.8 and 7.2, but in all cases the nitramide was almost spontaneously decomposed. To give some idea of the decomposition rate we give the following figures which, of course, have only an approximative value:

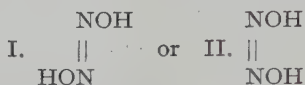
| pH  | $\mu$ L gas/min. |
|-----|------------------|
| 5.6 | 88               |
| 6.2 | 98               |
| 6.6 | 135              |
| 6.8 | 128              |
| 7.2 | 130              |

It is evident that with such rates of spontaneous decomposition in aqueous solution there is no possibility for making metabolic experiments<sup>1</sup>).

<sup>1</sup>) Note added in the proof: Recently P. W. WILSON and R. H. BURRIS report the same experience (cf. *Ann. Rev. Microbiol.* **7**, 427, 1953).

c. Discussion of the acceptability of hyponitrous acid and nitramide as intermediates in denitrification.

The idea that hyponitrous acid is an intermediate product in denitrification is very attractive, if one accepts the primary formation of the free radical  $=\text{NOH}$  in this process. For it seems highly acceptable that this radical by dimerization will lead to the formation of



Our experiments described under *a* show in full agreement with the experience of the earlier authors — ELEMA (1932); ALLEN and VAN NIEL (1952) — that the preparation known as sodium hyponitrite is unacceptable as an intermediate product in denitrification.

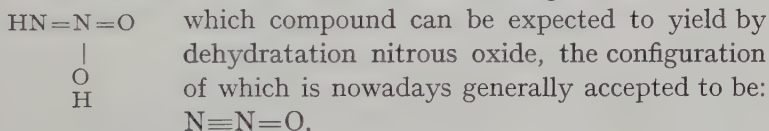
It is, therefore, important to discuss which configuration should be given to the compound in question. This has for a long time been a point of controversy between several authors: RASCHIG (1924), HUNTER and PARTINGTON (1933), HANTZSCH (1933), but for the moment the available evidence is in favour of the view that hyponitrous acid has the configuration I. This leaves the possibility that to the postulated intermediate product the configuration II should be ascribed.

This configuration seems to offer some advantages as a precursor of nitrous oxide (cf. HANTZSCH, 1933), but since at present its occurrence as an intermediate is not open to any experimental test no conclusion can be drawn.

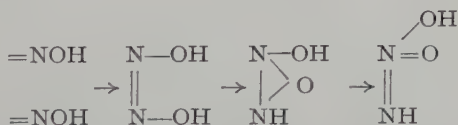
As for nitramide the following remarks should be made. In the first place we may not omit to point out the differences between the results obtained by ALLEN and VAN NIEL (1952) and our own. The American authors worked with a preparation which showed only a very slight spontaneous decomposition, whilst a vigorous gas evolution took place under the influence of the denitrifying bacteria. In contrast hereto our preparation was at once almost completely decomposed when brought into contact with a buffer solution.

This can only mean that different compounds have been tested in the two cases. It seems to be beyond doubt that our experiments have been conducted with the pure compound usually designated in literature as nitramide. On the other hand the American authors

have not been working with a pure compound; they used an acidified nitrocarbimate solution which immediately before use was brought to pH = 5.0, and next added to the bacterial suspension in phosphate buffer (pH = 7.0?). In view of the chemical studies of THIELE and LACHMAN (1894) a rapid decomposition of the nitrocarbimate and the resulting nitramide seems extremely likely under these conditions. We are, therefore, inclined to ascribe the metabolic changes observed by ALLEN and VAN NIEL to some compound different from nitramide. Our own results which demonstrate the great ease with which nitramide spontaneously decomposes into nitrous oxide and water make this compound a particular attractive precursor of nitrous oxide in denitrification. This is somewhat surprising, since the name of the compound suggests the configuration  $O_2NNH_2$ , and it is not easily understood how such a compound would lead to a formation of  $N_2O$ . However, here we meet with a case, in which a compound is not true to its name, for later investigators (HANTZSCH, 1933, HUNTER and PARTINGTON, 1933; FRIEDMAN and BIGEISEN, 1950) are more or less in favour of the view that the real configuration of the so-called „nitramide” is in fact imido-nitric acid, and that its real configuration is:



It is, therefore, very tempting to accept imido-nitric acid as an intermediate in denitrification. It should be acknowledged that its origin still remains obscure, but a mechanism like the following seems possible:



The acceptance of imido-nitric acid as an intermediate product in denitrification has on the one hand the advantage of yielding an easy explanation for the more or less capricious occurrence of nitrous oxide in this process, whilst on the other hand it is quite acceptable that continued hydrogenation interferes with the auto-decomposition of the intermediate, thus giving rise to the formation of molecular nitrogen and water.

## PART II.

IS NITROUS OXIDE A REGULAR PRECURSOR OF MOLECULAR NITROGEN  
FORMED IN DENITRIFICATION?a. Experiments on simultaneous enzymatic  
adaptation.

As has already been remarked in the Introduction SACKS and BARKER (1952) reject nitrous oxide as a normal intermediate in the formation of molecular nitrogen. This view is partly based on experiments on simultaneous enzymatic adaptation: cells of *Ps. denitrificans* which were adapted to nitrate only consumed nitrous oxide after a certain lag period. ALLEN and VAN NIEL who made analogous experiments with *Ps. stutzeri* did not observe this lag, and concluded that simultaneous adaptation, indeed, took place.

It seemed desirable to study this point also for our strains of *Ps. aeruginosa* and *M. denitrificans*.

In the experiments with *Ps. aeruginosa* the bacteria were cultivated on the peptone-nitrate agar medium as described under Part Ia. A suspension of these nitrate-adapted bacteria in the usual buffer were used for manometric experiments. In one vessel the side-arm contained 0.2 ml of a solution of 1 % glucose and 1 %  $\text{KNO}_3$ ; here air was replaced with oxygen-free nitrogen. In the second vessel the side-arm contained only 1 % glucose; here the apparatus was filled with nitrous oxide. The results of this experiment are reproduced in Fig. 3.

It will be seen that the graph dealing with the nitrous oxide does not show any lag so that one may conclude that indeed simultaneous adaptation has occurred. However, it should be acknowledged that the method used both by us and by the two groups of American investigators is far from accurate.

It should be realized that the amount of nitrogen evolved in the experiment with nitrous oxide as hydrogen acceptor is calculated from the relatively slight changes in pressure which result from the better solubility of nitrous oxide. This implies that small changes in pressure represent large quantities of nitrogen produced. The experiment shows that these are much larger than the amounts of nitrogen developed in the experiment with nitrate as hydrogen acceptor. This could more or less be expected, since 1 mol of nitrogen produced from nitrate asks for 10 H atoms, and 1 mol of nitrogen

produced from nitrous oxide only for 2 H atoms. It is remarkable that the ratio of the amounts of nitrogen produced in the two cases approaches the value 1 : 5, indicating that in both experiments the conversion rate is determined by the capacity of the dehydrogenase.

It is noteworthy that in the analogous experiments of ALLEN

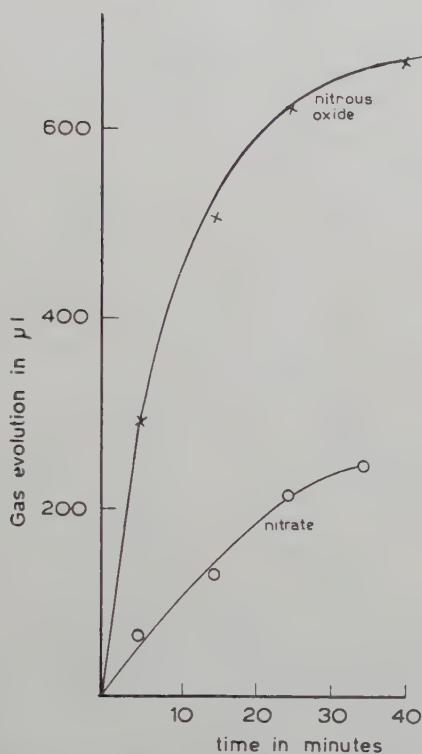


Fig. 3. Simultaneous enzymatic adaptation of *Pseudomonas aeruginosa* to nitrate and nitrous oxide as hydrogen acceptor.

Hydrogen donator: glucose. Phosphate buffer pH 7.2. Bacteria grown under anaerobic conditions on peptone agar, 1 %  $\text{KNO}_3$  and 0.5 % glycerol. The nitrate experiment in  $\text{N}_2$  atmosphere; in the nitrous oxide experiment the atmosphere initially was pure  $\text{N}_2\text{O}$ .

and VAN NIEL the said ratio is in the neighbourhood of 20, whilst in the experiments of SACKS and BARKER who compared the nitrogen evolved from nitrite and nitrous oxide, for which the theoretical ratio is 1 : 3, values of 1 : 0.13 were found.

It seems probable that these differences are largely due to the inaccuracy of the method of measuring nitrogen production from



nitrous oxide simply on the basis of the difference in solubility of the two gases.

We have, therefore, looked for a method of comparison of the two hydrogen acceptors more suitable to manometric measurement. A solution for this problem was found in our study on molecular hydrogen as a donator in denitrification. We had in the meantime established that "both nitrate- and hydrogenase-adapted" cells of *M. denitrificans* use molecular hydrogen readily in the presence of

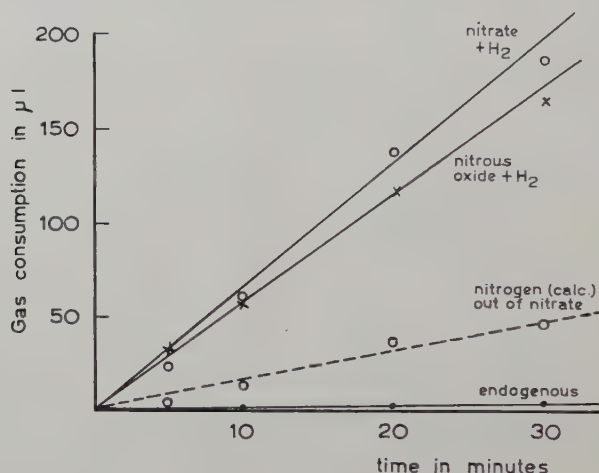


Fig. 4. Simultaneous enzymatic adaptation of *Micrococcus denitrificans* to nitrate and nitrous oxide as hydrogen acceptor.

Hydrogen donator: Molecular hydrogen. Phosphate buffer pH 7.2. Bacteria grown under anaerobic conditions on Grohmann agar (see text) with 1 %  $\text{KNO}_3$  and two drops of yeast autolysate. The nitrate experiment in  $\text{H}_2$  atmosphere; in the nitrous oxide experiment the atmosphere was initially 90 %  $\text{H}_2$  and 10 %  $\text{N}_2\text{O}$ .

nitrate as acceptor. It seemed worth-while to investigate how these cells would behave towards nitrous oxide as acceptor.

The bacteria were always cultivated under anaerobic conditions at 30° C. in a hydrogen atmosphere. The solid medium used for the cultivation of *M. denitrificans* consisted of 1 %  $\text{KNO}_3$ , 0.1 %  $\text{Na}_2\text{CO}_3$ , 0.1 %  $\text{NH}_4\text{Cl}$ , 0.07 %  $\text{KH}_2\text{PO}_4$ , 0.02 %  $\text{MgSO}_4$ , 0.02 %  $\text{NaCl}$ , a trace of  $\text{FeCl}_3$  and 2 % agar. It was necessary to add 2 drops of yeast autolysate to the agar in every Petri dish to get abundant growth. The 48 hours old cultures were further treated as indicated in Part Ia.

The experiments were repeated many times and yielded consistent results. The results of such an experiment which is representative for all is reproduced in Fig. 4.

In Fig. 4 the gas absorption as calculated from the observed pressure changes in the two cases are plotted. One should keep in mind that the amount of nitrogen evolved in the nitrate experiment is  $\frac{1}{4}$  of the gas absorption, whilst in the case of nitrous oxide the nitrogen evolved equals the observed absorption.

The dotted line gives the amount of nitrogen evolved from the nitrate, and shows that the ratio between this amount and that of the nitrogen evolved from the nitrous oxide is about 1 : 3. Apparently the theoretical value (1 : 5) on the basis of hydrogenase capacity as limiting factor of the rate of both conversions is not reached.

It should be kept in mind, however, that it is extremely difficult to observe a lag period in experiments in which only gases take part. For, in contrast to the usual procedure in Warburg experiments, the substrate is already added at the moment the vessel is placed in the thermostat. To minimize a possible adaptation period measurements were started as soon as possible after this operation, usually already after some minutes. The fact that no lag period was observed, combined with the linear course of gas consumption during the experiments itself, practically excludes an adaptation phenomenon. In contrast to the results of SACKS and BARKER (1952)<sup>1</sup>, but in agreement with those of ALLEN and VAN NIEL (1952), our apparently more reliable method of measurement also indicates that the nitrate-adapted bacteria are simultaneously adapted to nitrous oxide. There is, therefore, no reason to reject nitrous oxide as a regular intermediate in the conversion of nitrate into nitrogen on the basis of the principle of simultaneous enzymatic adaptation.

#### b. Experiments on the occurrence of nitrous oxide in denitrification.

In the Introduction it has been remarked that there seem to be two possibilities as regards the final step in denitrification, *i.e.* the formation of molecular nitrogen. In the first place it seems quite conceivable that nitrogen results from a direct hydrogenation of the postulated intermediate  $\text{N}_2\text{O}_2\text{H}_2$ . On the other hand we have

<sup>1</sup>) Note added in the proof: Dr BARKER kindly informed us that the lag was only observed with suspensions of cells derived from aged cultures.

seen that this intermediate is apparently easily decomposed into nitrous oxide and water. If this reaction should have preference, the resulting nitrous oxide will be the substrate of hydrogenation, and in acting as such will be converted into nitrogen.

The latter hypothesis is much more attractive, in so far as direct experimental proof is available that denitrifying bacteria can, indeed, use nitrous oxide as a hydrogen acceptor and convert it into nitrogen. This has already been observed by BEIJERINCK and MINKMAN (1910), and this has amply been confirmed by all later investigators who have deemed it worth-while to test this point.

Nevertheless, both SACKS and BARKER (1952) and ALLEN and VAN NIEL (1952) reject explicitly the possibility that nitrous oxide is a regular intermediate in denitrification. We will not consider here all the reasons for this view, but their attitude is at least partly determined by the circumstance that the former couple of investigators was unable to detect any nitrous oxide formation in their experiments, whilst the latter could only find very small quantities of nitrous oxide in the gas evolved.

These results contrast more or less with the experiences of VERHOEVEN (1952) for his sporeforming *Denitrobacillus licheniformis* which organism in the first phase of a culture experiment sometimes converted not less than 60 % of the nitrate-N into nitrous oxide, which gas then for the greater part was hydrogenated to molecular nitrogen in the second phase of the experiment. Moreover, it was shown that the ratio nitrous oxide: nitrogen in the total gas collected was highly influenced by changes in the partial pressure of nitrous oxide in the atmosphere in contact with the culture. These experiments taken together could in a way be considered as an "ad oculos"-demonstration of the intermediate nature of nitrous oxide.

Under these conditions it seemed desirable to extend these observations also to the more normal denitrifying bacteria, identical with or closely related to the species used by the American investigators.

For a first orientation experiments were made with seven strains belonging to three different species: *Pseudomonas aeruginosa*, *Micrococcus denitrificans* and *Pseudomonas stutzeri*. In these experiments the strains were grown in a 50 ml bottle completely filled with a medium containing 0.1 %  $K_2HPO_4$ , 0.05 %  $MgSO_4$  and varying amounts of peptone, potassium nitrate, glucose or glycerol, as

indicated in Table I. The gas evolved could escape at normal atmospheric pressure and was collected in a gas burette over mercury. After the gas evolution had stopped, the collected gas was submitted to analysis according to the method of MEYER and VOOGEL (1951). The results are collected in Table I.

TABLE I.

Nitrous oxide production by various bacteria under anaerobic conditions. Media: tap water, 0.1%  $K_2HPO_4$ , 0.05%  $MgSO_4$  and varying amounts of peptone, potassium nitrate, glucose or glycerol.

| species                 | strain number | addition to medium       | %KNO <sub>3</sub> | %N <sub>2</sub> O in gas |
|-------------------------|---------------|--------------------------|-------------------|--------------------------|
| <i>Ps. aeruginosa</i>   | 1             | 1% peptone 0.5% glycerol | 1.5               | 45.0                     |
| " "                     | 2             | 1% glucose               | 1                 | 7.9                      |
| " "                     | 2             | 1% peptone 1% glucose    | 1                 | 18.0                     |
| " "                     | 2             | 1% peptone 1% glucose    | 1                 | 20.0                     |
| " "                     | 3             | 1% glucose               | 1                 | 8.0                      |
| " "                     | 4             | 1% peptone               | 1                 | 0.0                      |
| " "                     | 4             | 1% peptone 1% glycerol   | 1                 | 5.5                      |
| " "                     | 4             | 1% peptone 1% glucose    | 1                 | 16.2                     |
| " "                     | 4             | 1% glucose               | 1                 | 12.8                     |
| " "                     | 4             | 1% glucose               | 1                 | 5.9                      |
| " "                     | 4             | 1% peptone 1% glucose    | 1                 | 13.7                     |
| <i>Ps. stutzeri</i>     | 1             | 1% peptone 1% glycerol   | 0.7               | 0.0                      |
| " "                     | 1             | 1% peptone 1% glucose    | 1                 | 14.0                     |
| <i>M. denitrificans</i> | 1             | 1% peptone 1% glucose    | 1                 | 24.2                     |
| " "                     | 1             | 1% peptone               | 1                 | 67.5                     |
| " "                     | 1             | 1% peptone 1% glycerol   | 1                 | 8.7                      |
| " "                     | 1             | 1% peptone 1% glucose    | 1                 | 32.3                     |
| <i>M. denitrificans</i> | 3             | 1% peptone 1% glycerol   | 0.7               | 14.0                     |
| " "                     | 3             | 1% peptone 2% glycerol   | 1.4               | 27.0                     |
| " "                     | 3             | 1% peptone 1% glucose    | 1                 | 36.7                     |

Although in two cases the collected gas did not contain any nitrous oxide, in all other cases this gas had been formed as a rule in quite considerable quantities. The most striking result is the large variation in the nitrous oxide contents of the gas even when one and the same strain is considered. This holds for instance for *M. denitrificans* strain 1: min. 8.7 %, max. 67.5 %. SIJDERIUS (1946) has already asked attention for this capricious behaviour of the said species. It is perhaps also noteworthy that in one case the strain



of *Ps. stutzeri* yielded no nitrous oxide at all, since this well identifiable species is the same as that used by ALLEN and VAN NIEL.

It is clear that these experiments in themselves do not constitute any proof that nitrous oxide is an intermediate in nitrogen formation, but they demonstrate clearly that all denitrifying bacteria tested are able to produce nitrous oxide.

It seemed, however, important to investigate in how far so-called "vacuum" experiments — in which the partial pressure of nitrous oxide in the atmosphere in contact with the culture is kept at a low value — always lead to a marked increase in nitrous oxide yield. These experiments were made with *Denitrobacillus licheniformis*, *Pseudomonas aeruginosa* and *Micrococcus denitrificans*. In these "vacuum" experiments a vacuum was intermittently established after each 4–6 hours cultivation, the experiment being completed within 20–24 hours. In each case a parallel experiment was run in which the gas could continuously escape at the normal pressure of the atmosphere. The culture medium contained peptone, glucose or glycerol, potassium nitrate and in some cases  $K_2HPO_4$  and  $MgSO_4$ . The initial pH was always adjusted to 7.2.

It may be remarked that within experimental error the total amount of gas evolved never surpassed the amount which can be expected to be formed from the nitrate consumed, proving that no nitrogen of the peptone is involved in the gas production. VERHOEVEN (1952) and also ALLEN and VAN NIEL (1952) already stressed this fact.

The results of the experiments are collected in Table II.

TABLE II.

The influence of the partial pressure of nitrous oxide on denitrification in anaerobic cultures of different bacterial species.

| species                               | strain number | nitrogen source | hydrogen donator | % $KNO_3$ | % $N_2O$ in gas evolved |        |
|---------------------------------------|---------------|-----------------|------------------|-----------|-------------------------|--------|
|                                       |               |                 |                  |           | vacuum                  | 1 atm. |
| <i>Ps. aeruginosa</i>                 | 1             | 1% peptone      | 1% glucose       | 1         | 58                      | 8      |
| <i>Ps. aeruginosa</i>                 | 1             | 1% peptone      | 0.5% glycerol    | 1.5       | 87                      | 37     |
| <i>Ps. aeruginosa</i>                 | 1             | 1% peptone      | 1% glucose       | 1         | 33                      | 8      |
| <i>Ps. aeruginosa</i> <sup>1)</sup>   | 2             | 0.5% peptone    | 1% glucose       | 1         | 80                      | 20     |
| <i>M. denitrificans</i> <sup>1)</sup> | 1             | 1% peptone      | 1% glucose       | 1         | 63                      | 24     |
| <i>Den. licheniformis</i>             | W             | 1% peptone      | 1% glucose       | 1         | 56                      | 14     |

<sup>1)</sup> In these experiments the medium also contained 0.1 %  $K_2HPO_4$  and 0.05 %  $MgSO_4$ .



These results leave not the slightest doubt that also the two common non-sporeforming denitrifying bacteria react on the lowering of the partial nitrous oxide pressure in exactly the same way as has earlier been described for *Denitrobacillus licheniformis*, viz., by a marked increase of nitrous oxide production. There is, therefore, all reason to conclude to an identical reaction mechanism for all denitrifying bacteria studied.

c. Discussion of the acceptability of nitrous oxide as an intermediate in denitrification.

As has already been remarked in the two preceding sections both SACKS and BARKER (1952) and ALLEN and VAN NIEL (1952) reject the possibility that nitrous oxide functions as an intermediate in denitrification. Although the two groups of investigators admit the non-stringent character of the argument, their attitude was at least partly determined by the fact that in their experiments the presence of nitrous oxide could not — or only exceptionally in small quantities — be established. However, SACKS and BARKER have two other arguments which, indeed, carry much more weight.

In the first place they conclude from their experiments that the bacteria under investigation do not show a simultaneous adaptation to nitrate and nitrous oxide: nitrate-adapted cells should only consume nitrous oxide after a measurable lag time. Secondly they found in sodium azide and dinitrophenol agents which in a certain low concentration completely inhibited nitrous oxide consumption, whilst these compounds in the same concentration did not affect the conversion of nitrite into molecular nitrogen.

ALLEN and VAN NIEL who carried out similar experiments with a different bacterial species came to a partly deviating conclusion, in so far as they found for their bacteria simultaneous adaptation to nitrate and nitrous oxide. However, their experiments yielded a confirmation of the fact that a cell poison, like cyanide can selectively block the nitrous oxide consumption, leaving the conversion of nitrite into nitrogen intact.

We shall now discuss these arguments against the acceptance of nitrous oxide as an intermediate in the light of the foregoing experiments.

In the first place we can then stress that the experiments described under *b* show clearly that the production of nitrous oxide in denitrification is not at all restricted to the special case of *Denitrobacillus*

*licheniformis* as described by VERHOEVEN (1952), but that also three of the most common non-sporeforming denitrifying bacterial species possess the ability to produce nitrous oxide. It is true that the ratio between nitrous oxide and nitrogen formed is liable to considerable variations — even to the degree that in rare cases nitrous oxide is absent —, but it is clear that these results far from justifying a rejection of the intermediate nature of nitrous oxide, must be considered favourable for the hypothesis in question.

Secondly we have made several experiments to decide whether the bacteria investigated by us showed simultaneous adaptation to nitrate and nitrous oxide, and in all cases we arrived at a positive result, in agreement with the experience of ALLEN and VAN NIEL. That SACKS and BARKER came to an opposite conclusion may well be due to the fact that they had to base their determination of this consumption only on the slight pressure changes resulting from the difference in solubility of nitrous oxide and nitrogen in the medium. Especially in the first phase of the experiment small variations in the experimental conditions might well influence the results. We have tried to avoid this difficulty by studying the said simultaneous adaptation with the aid of gaseous hydrogen as donator, owing to which gas exchange can be measured with much greater accuracy.

Taking all this together, we must conclude that the principle of simultaneous adaptation is in favour of the view that nitrous oxide is an intermediate.

We now arrive at the experiments of the American investigators dealing with the selective poisoning of the nitrous oxide hydro-generation under conditions which do not inhibit the conversion of nitrite into nitrogen. We have been able to confirm this result for the case that cyanide acts as a selective poison, and this demonstration seems to offer conclusive evidence that nitrous oxide is not always the immediate precursor of molecular nitrogen. At first there still seemed to be the possibility that an excess of nitrite might act as a detoxicating agent, especially since it is well-known that both cyanide and azide are successfully used for the removal of small quantities nitrite in water analysis. However, for the said reactions an acid medium is required, and we have been unable to prove even a partial removal of cyanide under the conditions of the manometric experiments, *i.e.*, in a phosphate buffer of pH = 7.2.

We are, therefore, prepared to accept that nitrous oxide is not an obligatory intermediate in the formation of molecular nitrogen in denitrification.

In the meantime this does not at all imply that under other conditions nitrous oxide does not act as an intermediate in denitrification. In a way VERHOEVEN (1952) has already conclusively proved that in cultures of *Denitrobacillus licheniformis* at a certain stage large amounts of nitrous oxide are in the medium which subsequently disappear under the formation of nitrogen. Here the intermediate character of nitrous oxide has, therefore, directly been shown.

In this respect also the so-called "vacuum-experiments" reported by VERHOEVEN for *Denitrobacillus licheniformis*, and extended in this paper to three other denitrifying bacterial species, seem to be highly significant.

The only possible interpretation of the fact that the simple measure of establishing a reduced partial pressure of nitrous oxide in the atmosphere in contact with the culture greatly enhances the nitrous oxide yield, seems to us that in all the cultures tested nitrous oxide was present in dissolved state in the medium.

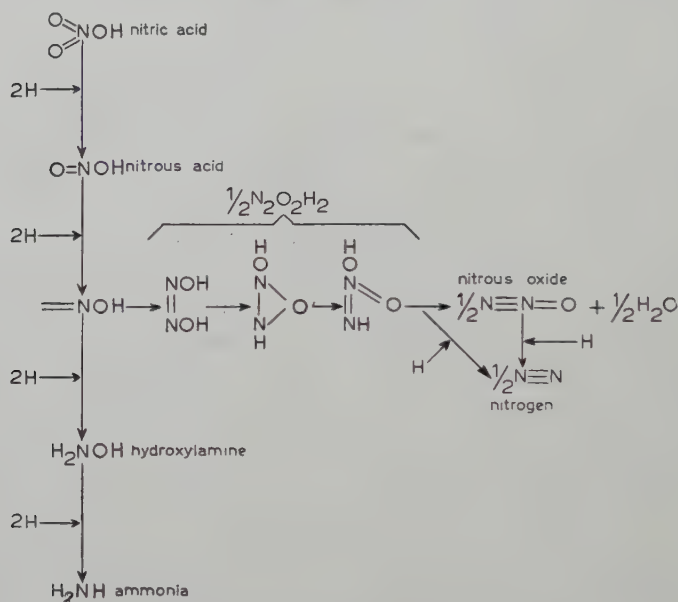
One might possibly object that the vacuum-effect might also be obtained, if the intermediate  $N_2O_2H_2$  which by hydrogenation yields nitrogen is liable to a reversible dissociation, the equilibrium of which is shifted to the nitrous oxide side by the reduced pressure. In this case the disappearance of preformed nitrous oxide would not be due to a direct hydrogenation, but to a resynthesis of  $N_2O_2H_2$ , and a subsequent hydrogenation of this compound.

However, we are inclined to reject this idea. The solubility of nitrous oxide in the alkaline region is lower than that at the neutral point, indicating the irreversibility of the decomposition of hyponitrous acid. Nor has the reversibility of any other reaction leading to a splitting off of nitrous oxide ever been shown. It is further remarkable that in spite of its specific physiological action (narcosis!), all chemical studies agree that nitrous oxide at room temperature is a very inert gas; it is for instance significant that the electric conductivity of an aqueous solution of nitrous oxide is the same as that of pure water.

On these grounds we are inclined to conclude from our experiments that nitrous oxide is an intermediate product in the normal denitrification process caused by various bacterial species, and as

a rule is for the greater part converted into nitrogen by hydrogenation. Yet this does not exclude that another part of the molecular nitrogen formed results from a direct hydrogenation of the intermediate product  $\text{N}_2\text{O}_2\text{H}_2$ , the immediate precursor of the nitrous oxide.

The results of all considerations — those of Part I included — can, therefore, adequately be expressed by the following scheme, essentially the same as those given in our earlier publications:



Scheme of dissimilatory nitrate reduction

### Summary.

A study has been made to test in how far nitrous oxide, hyponitrite and nitramide act as intermediate products in denitrification.

In Part I of the investigation it could first be shown that — in confirmation of the results obtained by ALLEN and VAN NIEL — hyponitrite did not act as a hydrogen acceptor in denitrification. It was then found that in an aqueous medium the rate of auto-decomposition of a pure preparation of nitramide into nitrous oxide and water was so high that it was impossible to demonstrate an acceleration of this conversion by the bacteria.



It was further pointed out that chemical studies have established that the compound known in literature as "nitramide" actually has the configuration of imido-nitric acid, and accordingly should be designated as such.

The observations referred to above, of course, do not exclude at all that imido-nitric acid is an intermediate in denitrification. To the contrary its acceptance as such has the advantage, that its properties seem apt to offer an explanation for the more or less capricious production of nitrous oxide in the denitrification process.

In Part II the general ability of the more common denitrifying bacteria to produce nitrous oxide was established; the amount of this gas formed proved, however, to be subject to large fluctuations. From comparative experiments in which analyses were made of the gases escaping from cultures of these bacteria, either at atmospheric pressure or at considerably reduced pressure, it was concluded that under normal conditions nitrous oxide regularly occurs as an intermediate.

Experiments in which it was tested whether *Pseudomonas aeruginosa* and *Micrococcus denitrificans* show a simultaneous enzymatic adaptation to nitrate and nitrous oxide confirmed the positive result obtained by ALLEN and VAN NIEL for *Pseudomonas stutzeri*. This outcome indirectly supports the acceptance of nitrous oxide as an intermediate. On the other hand the experiments of SACKS and BARKER and of ALLEN and VAN NIEL on the action of various cell poisons prove that nitrite can be decomposed without passing the nitrous oxide stage.

The conclusion is, therefore, drawn that the nitrogen produced in denitrification may have a dual origin: partly it is derived from a hydrogenation of nitrous oxide, partly it originates from a direct hydrogenation of the precursor of nitrous oxide; as such imido-nitric acid may well function.

#### A c k n o w l e d g m e n t .

The cooperation of Mr. H. HENSEL, Mr. LIE THIAM HOK, Mr. H. W. G. MEESTERS and Mr. A. A. QUE in the experimental part of this study is gratefully acknowledged.

#### R e f e r e n c e s .

- ALLEN, M. B. and VAN NIEL, C. B. 1952. J. Bact. **64**, 397.  
BEIJERINCK, M. W. mit Mitwirkung von MINKMAN, D. C. J. 1910. Centralbl. f. Bakt. II, **25**, 30.



- VON BRAUN, J. 1907. Stickstoff in: Abegg's Handbuch der anorganischen Chemie **3**, III, 38—239, Leipzig.
- BRÖNSTED, J. N. und PEDERSEN, K. 1924. Z. physik. Chem. **108**, 185.
- ELEMA, B. 1932. De bepaling van de oxydatie-reductie potentiaal in bacteriëncultures en hare betekenis voor de stofwisseling. Thesis, Delft.
- FRIEDMAN, L. and BIGELEISEN, J. 1950. J. Chem. Phys. **18**, 1325.
- HANTZSCH, A. 1933. Ber. **66**, 1566.
- HUNTER, E. C. E. and PARTINGTON, J. R. 1933. J. Chem. Soc. p. 309.
- KLUYVER, A. J. 1953. Some aspects of nitrate reduction. In: Symposium Microbial Metabolism. Roma.
- MARLIES, C. A., LA MER, V. K. and GREENSPAN, J. 1939. Inorganic syntheses. Booth, H. S., Editor. New York and London. I, 68.
- MEYER, G. and VOOGEL, P. 1951. Rec. trav. chim. **70**, 833.
- RASCHIG, F. 1924. Schwefel- und Stickstoffstudien. Leipzig-Berlin.
- SACKS, L. E. and BARKER, H. A. 1952. J. Bact. **64**, 247.
- SIJDERIUS R. 1946. Heterotrophe bacteriën die thiosulphaat oxyderen. Thesis, Amsterdam.
- THIELE, J. und LACHMAN, A. 1894. Ber. **27**, 1909.
- VERHOEVEN, W. 1952. Aerobic sporeforming nitrate reducing bacteria. Thesis, Delft.
-

(Institut Pasteur de Bruxelles).

## PREMIERS RESULTATS DE LA LYSOTYPYIE DE *S. PARATYPHI* B EN BELGIQUE

par

**J. BEUMER et A. VANDEWEYER-DEVEEN**

(Reçu le 4 Décembre 1953).

C'est en 1951 que fut décidée, avec l'accord du Dr A. FELIX, Directeur du Central Enteric Reference Laboratory and Bureau, la création à l'Institut Pasteur de Bruxelles d'un centre national de lysotypie de *S. typhi* et de *S. paratyphi* B <sup>1)</sup>.

Nous exposons ici les premiers résultats de la lysotypie des souches de *S. paratyphi* B, qui nous ont été envoyées par les Instituts Provinciaux d'Hygiène, du 1<sup>er</sup> octobre 1951 au 1<sup>er</sup> octobre 1953. A ce jour 96 souches, provenant de 83 foyers différents ont été typées, avec les résultats consignés dans le tableau I.

Nous avons suivi exactement la technique prescrite par le Central Enteric Reference Laboratory and Bureau (1, 2): elle ne nous a donné aucune difficulté particulière.

En 1952, nous avons envoyé au Dr FELIX huit souches que nous n'avions pas réussi à typer dans notre laboratoire. Quatre de ces souches appartenaient au type BAOR, dont nous ne possédions pas le phage-type à l'époque. Deux autres souches étaient du type 1; elles réagissaient faiblement aux phages-types 1 et 2 entre nos mains, mais avaient un comportement normal entre celles du Dr FELIX. Nous supposons que le lot de milieu que nous utilisions à l'époque était de qualité inférieure. Enfin les deux dernières souches, que nous n'avions pu typer, appartenaient en réalité au type 3aI et nous avons pu ultérieurement confirmer nous-mêmes ce diagnostic avec nos phages-types: nous ne nous expliquons pas l'erreur commise.

---

<sup>1)</sup> Nous exprimons au Dr FELIX notre gratitude pour l'amabilité avec laquelle il a mis à notre disposition les phages et les souches nécessaires, ainsi que pour les conseils qu'il nous a prodigués et l'aide qu'il nous a apportée dans l'identification des souches de type douteux.

TABLEAU I.

Types Vi des souches de *S. paratyphi* B isolées en Belgique.

| Types Vi        | Nombre<br>de<br>souches | Nombre<br>de<br>foyers | Pourcentage<br>de<br>foyers |
|-----------------|-------------------------|------------------------|-----------------------------|
| 1               | 9                       | 7                      | 8,43                        |
| 2               | 1                       | 1                      | 1,20                        |
| 3a              | 9                       | 8                      | 9,64                        |
| 3aI             | 11                      | 11                     | 13,25                       |
| 3b              | 2                       | 1                      | 1,20                        |
| Jersey          | 0                       | 0                      | 0                           |
| Beccles         | 16                      | 16                     | 19,27                       |
| Taunton         | 27 *)                   | 19                     | 22,89                       |
| BAOR            | 19                      | 18                     | 21,68                       |
| Dundee          | 2                       | 2                      | 2,41                        |
| Non identifiées | 0                       | 0                      | 0                           |
|                 | 96                      | 83                     | 99,97                       |

\*) Dont deux d'origine étrangère, dont il n'a pas été tenu compte pour le calcul des pourcentages des foyers situés en Belgique.

Les résultats obtenus sont donc satisfaisants, aucune de nos souches n'étant restée indéterminée. Signalons à ce propos l'utilité de la table des variantes des schémas de lysotypie de certains types, publiée par FELIX et CALLOW (2). Il est indispensable d'en tenir compte pour éviter des difficultés dans l'identification des souches dont le comportement n'est pas classique.

Les souches du type 3b sont parfois difficiles à identifier, en raison du caractère particulier de la lyse par le phage 3b. Cette lyse, dite "lyse opaque", est souvent difficile à distinguer de la trace laissée sur la gélose, en l'absence de toute action lytique, par la goutte de phage que l'on y a déposée. Nous avons eu recours avec succès à l'artifice suivant pour distinguer entre "lyse opaque" et absence de lyse. Nous déposons sur la même plaque de gélose ensemencée de la culture à typer, en même temps que la dilution classique d'emploi du phage 3b, deux gouttes de ce phage, l'une dix fois plus concentrée et l'autre dix fois plus diluée. En cas de "lyse opaque" le phage le plus dilué donne des taches séparées nombreuses, faciles à distinguer, indiquant par là que la dilution classique donne une lyse confluyente, voilée par la culture secondaire. En cas d'absence de lyse par la dilution classique, le phage le plus dilué ne donne

pas de taches distinctes, mais il peut se faire que le phage le plus concentré en donne quelques unes, ce qui démontre qu'à la dilution classique le phage ne donne pas de lyse confluyente.

Les fréquences relatives des différents types Vi de *S. paratyphi* B en Belgique ressortent du tableau I, où nous les avons exprimées en pourcentages du nombre total des foyers dénombrés, selon la recommandation du Central Enteric Reference Laboratory and Bureau.

Ce tableau nécessite quelques commentaires. La fréquence des différents types de *S. paratyphi* B, calculée sur 83 foyers identifiés en deux ans, n'a qu'une valeur relative et on ne peut en tirer que des conclusions prudentes quant à la connaissance des types les plus répandus en Belgique. En effet, bien que les statistiques officielles ne distinguent pas les différents types des fièvres paratyphoïdes déclarées, nous avons toutes raisons de croire que nous n'avons reçu aux fins d'identification qu'une partie des souches de *S. paratyphi* B responsables des cas de paratyphoïde en Belgique. Ce qui est plus grave, 67 sur les 83 foyers identifiés l'ont été dans la Province de Liège. Si la paratyphoïde est plus fréquente dans cette partie du pays, il faut noter toutefois que le nombre élevé de souches identifiées en provenance de la Province de Liège s'explique également par le fait que c'est l'Institut d'Hygiène de cette province (Dir. Dr P. FREDERICQ) qui nous a le plus régulièrement adressé des demandes d'identification.

Dans le tableau II nous avons comparé la fréquence relative des différents types Vi de *S. paratyphi* B en Belgique à ce qu'elle est dans les pays limitrophes. On constate qu'en Belgique trois types (Taunton, BAOR et Beccles), de fréquence sensiblement égale, se retrouvent dans 60 p. 100 des foyers identifiés; trois autres types (3aI, 3a et 1), moins fréquents, ont été identifiés dans 30 p. 100 des foyers. Il n'y a donc pas prédominance nette d'un type déterminé, les trois types les plus fréquents (Taunton, BAOR et Beccles) ne dépassant que de peu 20 p. 100 chacun. Notons enfin que c'est en Belgique que les types BAOR et Beccles paraissent être les plus fréquents.

La situation est très différente en France et en Angleterre où un type domine nettement tous les autres et se rencontre dans près de la moitié des foyers: en France 47,58 p. 100 des cas sont dus au type Taunton et en Grande-Bretagne on trouve le type 1

TABLEAU II.  
Fréquence des types Vi de *S. paratyphi* B en Belgique  
et dans les pays limitrophes.

| Types           | Belgique<br>(1951-53) | Pays-<br>Bas <sup>1)</sup><br>(1952) | Allemagne<br>Occ. <sup>2)</sup><br>(1950-53) | France <sup>3)</sup><br>(1950-52) | Grande-<br>Bretagne <sup>4)</sup><br>(1950-52) |
|-----------------|-----------------------|--------------------------------------|----------------------------------------------|-----------------------------------|------------------------------------------------|
| 1               | 8,43                  | 11,40                                | 10,83                                        | 3,0                               | 46,60                                          |
| 2               | 1,20                  | 0                                    | 0                                            | 0                                 | 8,80                                           |
| 3a              | 9,64                  | 6,90                                 | 12,23                                        | 0,43                              | 7,20                                           |
| 3aI             | 13,25                 | 16,20                                | 17,09                                        | 2,19                              | 2,20                                           |
| 3b              | 1,20                  | 0,90                                 | 3,81                                         | 0,14                              | 2,50                                           |
| Jersey          | 0                     | 7,30                                 | 0,23                                         | 19,47                             | 1,80                                           |
| Beccles         | 19,27                 | 12,40                                | 6,56                                         | 3,51                              | 3,60                                           |
| Taunton         | 22,89                 | 28,40                                | 32,50                                        | 47,58                             | 11,0                                           |
| BAOR            | 21,68                 | 10,0                                 | 5,58                                         | 0                                 | 0,90                                           |
| Dundee          | 2,41                  | 2,30                                 | 1,68                                         | 22,25                             | 4,80                                           |
| Non identifiées | 0                     | 4,10                                 | 11,12                                        | 1,31                              | 10,60                                          |

<sup>1)</sup> R. TH. SCHOLTENS, Rapport au 6ème Congrès Intern. Microbiol. Rome 1953.

<sup>2)</sup> H. SCHLOSSBERGER, ibidem.

<sup>3)</sup> P. NICOLLE, ibidem.

<sup>4)</sup> Central Enteric Reference Laboratory and Bureau, London. ibidem.  
N.B. La fréquence des différents types est exprimée en pourcentage des foyers, sauf pour la France où les chiffres indiquent des pourcentages du nombre de cas.

dans 46,6 p. 100 des foyers. La répartition des types en France diffère encore de celle rencontrée en Belgique par la fréquence relative des types Dundee et Jersey, dont le premier est rare et le second inexistant jusqu'à présent dans notre pays. On relève des différences semblables entre Belgique et Grande-Bretagne, des types relativement fréquents dans ce dernier pays (type 2 p. ex) étant rares en Belgique et inversement (type BAOR p. ex).

L'examen de la répartition des types en Allemagne Occidentale montre que les six types qui se retrouvent dans 90 p. 100 des foyers en Belgique, ont été identifiés dans 85 p. 100 des foyers en Allemagne Occidentale. Mais l'ordre de fréquence des types n'est pas le même dans les deux pays: Taunton, 3aI, 3a, 1, Beccles et BAOR en Allemagne et Taunton, BAOR, Beccles, 3aI, 3a et 1 en Belgique.

C'est, semble-t-il, avec la fréquence relative des types aux Pays-Bas, que la répartition en Belgique offre le plus de traits communs.



Sept types se rencontrent aux Pays-Bas dans plus de 90 p. 100 des foyers; ces types sont, par ordre d'importance: Taunton, 3aI, 1, Beccles, BAOR, Jersey et 3a. Cette répartition se rapproche à la fois de celle existant en Allemagne Occidentale et de la fréquence relative des types en Belgique. Toutefois la répartition des types aux Pays-Bas se rapproche davantage de celle rencontrée en Belgique, à cause de la fréquence relative dans ces deux pays des types BAOR et Beccles, comparativement rares ailleurs; elle s'en distingue cependant, notamment par l'existence du type Jersey, non encore isolé en Belgique.

La situation existant aux Pays-Bas, en Allemagne Occidentale et en Belgique diffère donc radicalement de celle régnant en France et en Grande-Bretagne. Alors que dans ces deux derniers pays, un type prend nettement le pas sur tous les autres, les souches isolées en Belgique et chez ses voisins du Nord et de l'Est appartiennent à plusieurs types différents, de fréquences très comparables et sensiblement les mêmes dans les trois pays. Sans vouloir fournir de cet état de choses une explication définitive, il est permis de supposer que dans ce dernier cas, la situation géographique de la Belgique, des Pays-Bas et de l'Allemagne Occidentale, situées dans les bassins de trois fleuves contigus et parfois confondus, ainsi que la fréquence des relations de voisinage rendent compte dans une certaine mesure de la diversité des types rencontrés et de leur répartition dans ces trois pays.

En terminant, signalons que dans le petit nombre de cas où nous avons pu recueillir des renseignements cliniques sur les souches identifiées, il est apparu que chez un même sujet les bacilles isolés à plusieurs reprises au cours de la maladie ou de la convalescence conservaient leur type bactériophagique. D'autre part, les bacilles isolés chez des sujets différents dans un même foyer ou chez des malades contaminés à la même source appartenaient au même type. Ceci confirme la valeur épidémiologique de la méthode de lysotypie par les phages Vi.

### R é s u m é.

En deux ans, 94 souches de *S. paratyphi* B isolées de 83 foyers situés en Belgique ont été identifiées par le Centre de Lysotypie de l'Institut Pasteur de Bruxelles. La détermination des types bactériophagiques s'est faite sans difficultés majeures, en tenant

compte de l'existence de variantes au sein de certains types. Une technique est décrite permettant de résoudre les difficultés que l'on rencontre dans l'identification de la souche 3b, en raison de l'existence de lyse opaque. Les différents types se rencontrent en Belgique avec des fréquences différentes de celles observées dans les pays limitrophes; la répartition des types en Belgique se rapproche de celle observée en Hollande, sans toutefois être identique. La constance du type des cultures isolées chez un même malade ou dans un même foyer confirme l'utilité de la méthode.

### Bibliographie.

1. FELIX, A. et CALLOW, B. R. 1943. Brit. Med. Bull. **2**, 127.
  2. FELIX, A. et CALLOW, B. R. 1951. Lancet **261**, 10.
-

(Institute of Microbiology, School of Medicine, University of Zagreb; Central  
Institute of Hygiene, Zagreb, Yugoslavia).

## DIFFERENTIATION OF *CORYNEBACTERIUM* *DIPHThERIAE* OF THE *MITIS* TYPE FOUND IN DIPHThERIA AND OZAENA

### I. BIOCHEMICAL PROPERTIES

by

VLADIMIR BEZJAK

(Received January 18, 1954).

BELFANTI and DELLA VEDOVA (1896) were the first to notice the occurrence of *Corynebacteria* in the nasal discharge of patients with ozaena (atrophic chronic rhinitis). These organisms were usually atoxic but the authors could not otherwise distinguish them from *Corynebacterium diphtheriae*. Regular presence of this *Corynebacterium* in the nasal secretions of ozaena was confirmed by SONNENSCHN (1925, 1926) and MIMICA *et al.* (1950). Some authors [STEIN (1900), ABEL (1928), COGLIA (1946)] did not distinguish this atoxic *Corynebacterium* from other saprophytic *Corynebacteria*.

Several studies in our institute since 1948 showed that a *Corynebacterium* with all the characters of *C. diphtheriae* type *mitis* can regularly be found in the nose of ozaena patients. We came to the conclusion that *Corynebacteria* may play a role in causing ozaena [MIMICA (1950), MIMICA *et al.* (1950), MIMICA and FRANKOVIĆ (1951), MIMICA and BEZJAK (1951)]. MIMICA *et al.* (1950) have isolated 75 strains of this *Corynebacterium* which was never toxigenic, but appeared to be pathogenic when injected intracerebrally into mice. The same observation was made by FROBISHER (1947) with atoxic *C. diphtheriae* strains isolated from diphtheria cases.

No objective difference could be established between this organism and an atoxic *C. diphtheriae* type *mitis* isolated from diphtheria. Nevertheless, we used the name *Corynebacterium belfanti* merely to denote the *mitis* type of *C. diphtheriae* isolated from cases of ozaena.

During our routine work on *Corynebacteria* some differences between the two types of organisms were observed on which we will report now.

#### MATERIALS AND METHODS.

Initially three strains of *C. diphtheriae* type *mitis* isolated from diphtheria cases were compared with three strains of the so-called *C. belfanti* isolated from typical cases of ozaena in which *Klebsiella ozaenae* has also been found.

We examined the morphological, cultural, biochemical and pathological properties according to the methods described by MIMICA *et al.* (1950), except pathogenicity in mice. Additional methods for the identification of hitherto undescribed strains were used, following the table of FROBISHER (1947). No serological tests were used.

The method for studying nitrate reduction was as follows: The medium consisted of Bacto beefextract 3‰, Bacto-peptone 5‰, potassium nitrate 1‰, Bacto agar 12‰. After 24 hours incubation the cultures were flooded with  $\alpha$ -naphthylamin and sulfanilic acid. The appearance of a red colour indicated the reduction of nitrates.

After the preliminary results a larger number of strains was tested with regard to nitrate reduction: 42 strains of *C. diphtheriae* type *mitis* and 52 strains of *C. belfanti*. Of the latter strain 47 were isolated from ozaena patients, five however were isolated from the nose of normal people during a search for *C. diphtheriae* in human carriers.

#### RESULTS.

The comparison between the strains of *C. diphtheriae* type *mitis* isolated in diphtheria and of those found in ozaena patients revealed the only difference in the reduction of nitrates. In other respects i.e. in morphology, colonial appearance and other biochemical reactions no differences could be observed. All the strains were negative when tested in guinea-pigs or rabbits.

All of 45 strains of *C. diphtheriae* type *mitis* isolated in diphtheria patients or carriers reduced nitrates whereas the 55 strains isolated in ozaena patients were negative as to this reduction.

The five strains of *Corynebacteria* isolated from suspected

diphtheria carriers were classified as *C. belfanti* because they did not reduce nitrate. At our request a rhinologist examined the nasal cavities of these children. In each of them his diagnosis was atrophy of nasal mucosa.

Of ten additional strains of *C. diphtheriae* type *mitis* obtained from the Institute of Microbiology, University of Ljubljana, three were negative as to nitrate reduction. We classified them as *C. belfanti* and we have been informed later that these strains had been isolated from ozaena patients in Ljubljana.

Three standard strains of *C. diphtheriae* type *mitis* from the Communicable Disease Center, Chamblee, Georgia all reduced nitrates readily.

Among the 50 cases of ozaena from which we isolated our strains of *C. belfanti*, *Klebsiella ozaenae* was found in only 36 instances or 72%.

#### DISCUSSION.

The results of this study indicate that *C. diphtheriae* type *mitis* found in diphtheria patients or carriers and the *Corynebacterium* isolated from ozaena patients are not identical. There exists a constant difference between them in the capacity to reduce nitrate. This only difference, however, does not permit us to make any decision on the taxonomic position of the so-called *C. belfanti*. Further examinations of the pathology, serology and epidemiology of this *Corynebacterium* are necessary. Such studies are already in progress and the results will be published in due course.

#### S u m m a r y.

The purpose of this study was to find out whether there existed any difference between *C. diphtheriae* type *mitis* always present in the nasal cavity of ozaena patients (the so-called *C. belfanti*) and *C. diphtheriae* of the *mitis* type found in diphtheria patients or carriers.

Studying in details all the morphological, cultural and biochemical properties, a difference was found to exist in the reduction of nitrates. This test was investigated in 55 strains of *C. belfanti* and 45 strains of the *mitis* type of *C. diphtheriae*. All the strains of *C. belfanti* yielded negative results in the reduction of nitrates, while



all strains of *C. diphtheriae* type *mitis* reduced nitrates within 24 hours <sup>1)</sup>).

The value of this observation was shortly discussed.

### A c k n o w l e d g m e n t .

I wish to express my gratitude to Dr HEDA DUBAC and Miss MAGDA ROSENBERG who have carried out much of the technical work; to Professor MILICA VALENTINČIĆ and Dr ELISABETH I. PARSONS who supplied me with some diphtheria strains; to Professor WINKLER who brought the paper in more suitable shape; and finally to all colleagues who helped me in this study.

### R e f e r e n c e s .

- ABEL, R. 1929. Handb. d. Path. Mikroorg. (Kolle, Kraus, Uhlenhuth). Fischer-Urban-Schwarzenberg, Jena. Vol. VI/1.
- BELFANTI, S. and DELLA VEDOVA. 1896. Giorn. Acad. med. Torino **2**, 149.
- COGLIA, L. 1946. Annali d'Igiene No 6.
- FROBISHER, M., PARSONS, E. I. and UPDYKE, E. 1947. Am. J. Publ. Health **37**, 543.
- FROBISHER, M. 1946. Fundamentals of Bacteriology. Saunders, Philadelphia-Londen.
- MIMICA, M. 1950. Acta med. Iugoslav. **4**, 276.
- MIMICA, M., UJEVIĆ, B. and MADJARIĆ, I. Acta. med. Iugoslav. **4**, 293.
- MIMICA, M. and FRANKOVIĆ, A. 1951. Acta med. Iugoslav. **5**, 95.
- MIMICA, M. and BEZJAK, V. "Rad" Acad. Yougoslav. 1951. **284**, 71.
- SONNENSCHN, C. 1925. Münch. med. Wschr. **72**, 1488.
- SONNENSCHN, C. 1926. Centralbl. f. Laryng. Rhinol. **14**, 450.
- STEIN, W. 1900. Centralbl. f. Bakt. I. **28**, 726, 769.

---

<sup>1)</sup> After the study was finished we have been able to test another 40 strains of Corynebacteria, i.e. 22 of *C. belfanti* and 18 of *C. diphtheriae* type *mitis*. The reduction of nitrates in all strains corresponded to the results given above.

(Laboratory of Microbiology, Technological University, Delft, Holland).

## STUDIES ON TRUE DISSIMILATORY NITRATE REDUCTION

### III. *MICROCOCCUS DENITRIFICANS* BEIJERINCK, A BACTERIUM CAPABLE OF USING MOLECULAR HYDROGEN IN DENITRIFICATION <sup>1)</sup>

by

W. VERHOEVEN, A. L. KOSTER and M. C. A. VAN NIEVELT

(Received February 5, 1954).

#### 1. INTRODUCTION.

In an earlier publication VERHOEVEN and GOOS (1954) have reported on a study of the fate of the hydrogen donator in true dissimilatory nitrate reduction. In this investigation only organic hydrogen donators have been considered.

In view of the claims that both carbonate reducing bacteria (SÖHNGEN, 1906) and sulphate reducing bacteria (STEPHENSON and STICKLAND, 1931 *b*) are able to thrive at the expense of the system inorganic hydrogen acceptor and molecular hydrogen, it seemed worth-while also to make a study of the suitability of this particular hydrogen donator in true dissimilatory nitrate reduction (VERHOEVEN, 1952; KLUYVER, 1953).

In literature several observations are found which leave no doubt that a bacterial reduction of nitrate with the aid of molecular hydrogen does occur under certain conditions. A critical examination of these data seems appropriate in order to answer the question whether these observations do, indeed, apply to true dissimilatory nitrate reduction.

Taking into consideration that all bacteria which are characterized by a true dissimilatory nitrate reducing ability are facultative aerobes, or in other words that they are able to interchange nitrate and free oxygen as hydrogen acceptors, it is easily understood that

---

<sup>1)</sup> Part I and II: *Antonie van Leeuwenhoek* **20**, 93, 241, 1954.

the first observations regarding the point in question have been made by investigators who studied the so-called "Knallgasbakterien".

KASERER (1906) makes the more or less incidental remark that his *Bacillus pantotrophus* which was able to oxidize molecular hydrogen with oxygen, did not do so with nitrate. In contrast herewith LEBEDEFF (1909) established that in cultures of bacteria oxidizing molecular hydrogen with oxygen the nitrate present in the medium was also reduced and partly converted into gaseous nitrogen. The subject was more systematically studied by NIKLEWSKI who in several publications (cf. NIKLEWSKI, 1914) gave a convincing demonstration that his *Hydrogenomonas agilis* is able to reduce nitrate with molecular hydrogen under formation of nitrogen. A remarkable, but only poorly documented statement is that *H. agilis* is unable to reduce nitrate with the aid of organic compounds, although this bacterium is quite able to grow heterotrophically as far as free oxygen is available. This implies that *H. agilis* does not belong to the common denitrifying bacteria.

In his thorough study of the "Knallgasbakterien" GROHMANN (1924) states explicitly that he was unable to establish the existence of bacteria which were able to develop in a medium in which the system molecular hydrogen and nitrate was the only source of energy. RUHLAND (1924) in his fundamental publication on the same subject, also reports that all "Knallgasbakterien" isolated by him were unable to oxidize hydrogen with nitrate instead of free oxygen.

The rather general belief that molecular hydrogen can, indeed, be used in bacterial nitrate reduction goes back to the investigation of STEPHENSON and STICKLAND (1931 *a*) who showed that "resting cells" of several strains of *Bacterium coli* are able to reduce nitrate with molecular hydrogen to nitrite. It is clear that this is a typical case of what we have called incidental dissimilatory nitrate reduction. There is not the slightest indication that the system molecular hydrogen/nitrate can act as the sole source of energy for the organism in question, and this may even be deemed extremely unlikely, since *B. coli* is neither enabled to a true dissimilatory nitrate reduction with organic hydrogen donators, nor to a life based on the oxidation of molecular hydrogen with oxygen.

Exactly the same remarks apply to the study of WOODS (1938) who established that "resting cells" of *Clostridium welchii* are

able to reduce nitrate with molecular hydrogen to the ultimate reduction product, ammonia.

The observations of LEWIS (1951) who proves that molecular hydrogen can act as a hydrogen donator in nitrate reduction by a mixture of unidentified rumen micro-organisms, are apparently another demonstration of incidental dissimilatory nitrate reduction.

SCHATZ and BOVELL (1951) describe that "resting cells" of their *Hydrogenomonas facilis* are able to reduce nitrate to nitrite with molecular hydrogen. The explicit statement that this bacterium is a strictly aerobic organism warrants the conclusion that here again we are dealing with a case of incidental dissimilatory nitrate reduction.

Finally mention should be made of the recent publication by SCHLEGEL (1953) who established that "resting cells" of his *Hydrogenomonas* species produce nitrite from nitrate with the aid of molecular hydrogen.

From the survey given it results that there is no unambiguous evidence that there exist bacteria which are able to proliferate in media in which the system nitrate/molecular hydrogen acts as sole source of energy. In order to test this point experimentally it seemed indicated to examine first in how far the better known denitrifying bacterial species are willing to accept molecular hydrogen as a substitute for the usual organic hydrogen acceptors.

For a preliminary orientation it appeared convenient to study the behaviour of "resting cells" of these bacteria in media containing nitrate in contact with a hydrogen atmosphere. If under these conditions nitrate reduction would occur, a further investigation should answer the question whether the said system can meet the energy requirement of the organism.

## 2. EXPERIMENTS ON "RESTING CELLS" OF PURE CULTURES.

Manometric experiments were made with "resting cells" of the following typical denitrifying bacteria: *Pseudomonas denitrofluorescens*, *Pseudomonas stutzeri*, *Micrococcus denitrificans*, *Pseudomonas aeruginosa* and *Denitrobacillus licheniformis*.

From earlier manometric studies on denitrification by VAN OLDEN (1940) it has resulted that "nitrataase", the nitrate reducing enzyme, is a typical adaptive enzyme, so that "resting cells" only reduce nitrate without considerable lag, if they have been grown in a nitrate containing medium. In addition KLUYVER and MANTEN

(1942) have proved that an analogous situation exists in bacteria oxidizing hydrogen with oxygen, in so far that these bacteria only contain hydrogenase, if they are cultivated in a hydrogen containing atmosphere.

For these reasons the various denitrifying bacteria tested in the "resting cell" experiments were all grown in a hydrogen atmosphere on an agar medium containing 1% peptone and 1—2% potassium nitrate.

The main vessel of the Warburg apparatus contained 1.5 ml of a cell suspension in 1/15 mol phosphate buffer pH = 7.2, the side bulb 0.2 ml of 1% potassium nitrate solution. The vessel was filled with oxygen-free hydrogen.

With a view to the results obtained by VAN OLDEN (1940) in his denitrification experiments with "resting cells" and organic hydrogen donators one might expect, that, if nitrate would also be reduced with molecular hydrogen, the following reaction would occur:



which would imply a considerable reduction in pressure.

The results obtained in these experiments were throughout negative. The relatively small pressure changes observed were practically the same whether or not nitrate had been added to the bacterial suspension, and whether the atmosphere consisted of hydrogen or of nitrogen.

The negative outcome of these experiments strongly suggested that, if bacteria exist which can bring about a denitrification with molecular hydrogen, they are of a specific type, and do not belong to the group of better known denitrifying species.

### 3. ENRICHMENT CULTURES OF DENITRIFYING BACTERIA USING MOLECULAR HYDROGEN AS HYDROGEN DONATOR.

It was then decided to start enrichment cultures in a medium containing nitrate and a few other essential mineral salts under conditions in which molecular hydrogen was the sole hydrogen donator present. This implies the absence of all organic compounds, so that in these media only autotrophic bacteria could develop.

Some garden soil was inoculated into a somewhat modified Eldredge tube containing 200 ml of a mineral medium as prescribed by GROHMANN (1924) which has the following composition:



0.1%  $\text{Na}_2\text{CO}_3$ , 0.02%  $\text{MgSO}_4$ , 0.1%  $\text{NH}_4\text{Cl}$ , 0.02%  $\text{NaCl}$ , 0.07%  $\text{KH}_2\text{PO}_4$  and a trace of  $\text{FeCl}_3$ . To this medium 2%  $\text{KNO}_3$  was added;  $\text{pH} = 7.2 - 7.5$ . The air in the remaining space of the vessel — about 200 ml — was replaced by a gas mixture consisting of 15%  $\text{CO}_2$  and 85%  $\text{H}_2$ . The culture vessel was continuously shaken in an incubator room at  $30^\circ \text{C}$ .

After two to three days a considerable reduction of gas pressure could be established. In several cases in which only pure hydrogen was used for replacing the air the decrease in pressure approached the value of  $4/5$  atmosphere, the value to be expected on the basis of the equation given in § 2 in the case that all hydrogen had been consumed. Attempts were made to isolate the bacteria responsible for the hydrogen absorption on a mineral agar medium of the same composition as that used in the enrichment experiment with the exception that this time tap water was used instead of distilled water. On cultivating in the hydrogen carbon dioxide atmosphere, however, no growth occurred on the plates. It was further noteworthy that also transfers directly from the enrichment culture into fresh liquid media of the same composition quickly lost their activity.

These so disappointing results strongly indicated that the bacteria which did grow in the original enrichment culture were no strict autotrophs. It seemed possible that we were dealing here with a case of chemo-metatrophs (LWOFF, 1943), that is the case in which the energy is derived from an inorganic conversion, whilst nevertheless organic growth substances are indispensable for the proliferation of the organism.

In this trend of thought transfers from the enrichment culture were made into a similar medium to which 0.3% yeast autolysate had been added. These transfers were quite successful, and moreover by plating on a mineral agar to which the same amount of yeast autolysate had been added a large number of a uniform type of colonies was obtained. It proved then to be an easy task to isolate a pure culture of the organism. Examination showed this to be quite active in the original liquid medium, provided that again a small quantity of yeast autolysate was added.

#### 4. PROPERTIES AND DESCRIPTION OF THE ISOLATED BACTERIUM.

A study of the pure culture showed at once that the organism was quite able to grow heterotrophically under aerobic conditions,

in the absence of both nitrate and hydrogen. Moreover, it brought about an active denitrification in a medium containing 1%  $\text{KNO}_3$ , 1% peptone and 1% glucose.

To test the suitability of various hydrogen donors the isolated bacterium was uniformly suspended in test tubes containing a high layer of washed agar to which 1%  $\text{KNO}_3$  and 1% of the substance to be tested had been added. In all cases a control tube containing washed agar with the organic substrate, but no nitrate, was also inoculated. If in the first tube cracking of the agar occurred, and if this was absent in the control tube, it was concluded that the substrate in question was able to function as hydrogen donor in denitrification.

The following substrates were found to be positive:

|                |          |             |            |
|----------------|----------|-------------|------------|
| ethyl alcohol  | glycerol | formiate    | asparagine |
| propyl alcohol | mannitol | acetate     |            |
|                | glucose  | lactate     |            |
|                |          | succinate   |            |
|                |          | fumarate    |            |
|                |          | citrate     |            |
|                |          | valerianate |            |

Though the organism proved to be able to grow in a liquid medium in which a suitable hydrogen donor is the only organic substance, as a rule the addition of some drops of yeast autolysate considerably accelerated the development.

Microscopic examination of 36 hours old cultures showed preponderantly an immotile coccus of  $1\ \mu$  in diameter, though as a rule also some spindle shaped cells and even rod forms can be observed. Sometimes also aggregates resembling a bunch of grapes were observed. In four days old cultures all our isolates invariably showed mainly the typical coccus form.

Fig. 1 gives a micrograph of such a culture after staining with carbol fuchsin.

The bacteria are Gram—. The colonies on peptone agar are relatively small, smooth and white. In broth a turbid growth is obtained. The organism does not liquefy gelatin.

In some experiments — partly already reported by KLUYVER and VERHOEVEN (1954) — in which the gas evolved from a culture in 1% peptone, 1% glucose, 1%  $\text{KNO}_3$  was collected, it could be established that after removal of the carbon dioxide the gas consisted of a mixture of nitrogen and nitrous oxide. It further ap-

peared that the nitrate nitrogen was quantitatively recovered in the nitrogen nitrous oxide mixture.

All these properties made it tempting to identify the organism with the bacterium briefly described by BEIJERINCK (1910) as *Micrococcus denitrificans*.

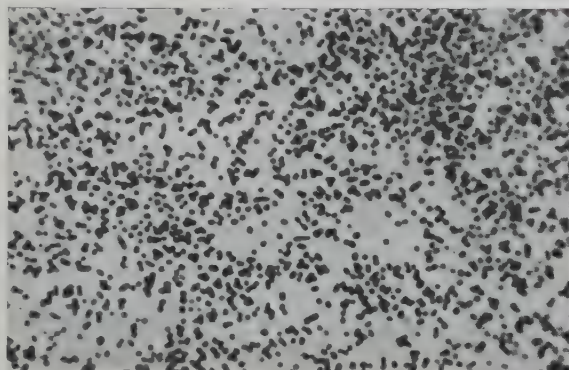


Fig. 1. *Micrococcus denitrificans* Beijerinck, 4 days old. Stained with carbol fuchsin. Magnification 1000  $\times$ .

As far as we have been able to ascertain the only other references in literature to this species are those made by ELEMA (1932) and by SIJDERIUS (1946)<sup>1</sup>. ELEMA gives a description of the bacterium in a footnote on page 110 of his thesis, which description is based on an authentic culture of BEIJERINCK maintained in the culture collection of the Laboratory of Microbiology at Delft.

SIJDERIUS reports that he encountered *M. denitrificans* in his

<sup>1</sup> Dr R. S. BREED at Geneva (N.Y.) U.S.A. was so kind as to draw our attention to the publication of ROBINSON and GIBBONS (1952) in which these authors describe a halophilic denitrifying bacterium for which they propose the name *Micrococcus halodenitrificans*. Judging from the description of this species as given by GIBBONS for the 7th Edition of Bergey's Manual of Determinative Bacteriology (in press) — which Dr BREED was so kind to forward to us — there seems to be reason to consider the new species as a salt-adapted strain of BEIJERINCK's species the main differences being the facts that the new strain requires at least 4% NaCl in its culture medium and that it liquefies gelatin. As for the salt adaptation it is worth mentioning that HOF (1935) reports that BEIJERINCK's strain still developed in media with 6% NaCl, and in a medium with 3% NaCl even faster than in a medium without salt. Evidently the strains isolated by the Canadian investigators have become obligately halophilic.

denitrification experiments with inorganic sulphur compounds, and concludes that the bacterium is closely related to *Thiobacillus novellus* Starkey, which he considers as a variety of *M. denitrificans*. SIJDERIUS gives a detailed description of the species which is also based on observations on BEIJERINCK's authentic culture. It is unnecessary to repeat here SIJDERIUS' results, since these are in almost perfect agreement with the properties of our new isolates, as outlined above.

We have also examined BEIJERINCK's authentic culture, and can agree with the descriptions as given by ELEMA and by SIJDERIUS.

There was, however, one reason which made us hesitate to conclude to the identity of our isolates with *M. denitrificans*. As reported in Section 2 "resting cells" of BEIJERINCK's strain had proved to be unable to reduce nitrate with molecular hydrogen in the manometric experiments, and at first sight this result seemed to contrast strongly with the active hydrogen consuming ability which was directly responsible for the isolation of our strains.

It was, therefore, a pleasant surprise when we could establish that also BEIJERINCK's more than forty years old strain, developed favourably in the mineral nitrate medium in contact with a hydrogen atmosphere, provided that some yeast autolysate was added to the medium. Under these conditions hydrogen was actively consumed.

Herewith the identification of our isolates with *M. denitrificans* Beijerinck may be considered to be fully justified.

##### 5. MOLECULAR HYDROGEN AS HYDROGEN DONATOR IN DENITRIFICATION BY PURE CULTURES OF *Micrococcus denitrificans*.

The facultative chemo-metatrophic character of *M. denitrificans* once having been established, it became quite easy to study the denitrification with molecular hydrogen in somewhat more detail.

Eldredge tubes with Grohmann medium with 2%  $\text{KNO}_3$  and a drop of yeast autolysate were again inoculated with *M. denitrificans* strain 5 and hereupon the remaining air was replaced by pure hydrogen. In a series of experiments a decrease in pressure of 4/5 atmosphere could regularly be established, indicating a total consumption of the hydrogen present.

In another series of quantitative experiments in which the amount of nitrate had been so restricted that hydrogen was present



in excess, it was found that the hydrogen consumed fully corresponded with a reduction of the nitrate to molecular nitrogen.

There remained, however, one point which still was in want of elucidation. We have reported in Section 2 that "resting cells" of BEIJERINCK's strain of *M. denitrificans* did not consume any hydrogen in a nitrate containing medium. This result appeared especially surprising, since the bacteria had been grown on a peptone medium containing 1% nitrate, and in a hydrogen atmosphere. This seemed to guarantee that the conditions for the production of both nitrataze and hydrogenase had been fulfilled.

In contrast hereto growing cultures of the same strain in a Grohmann medium with some yeast autolysate actively consumed hydrogen. It was, therefore, indicated to make a comparative test of "resting cells" grown on the two media.

These experiments were done with various strains of *M. denitrificans*, among which was also BEIJERINCK's strain. In all cases they yielded the same results.

Petri dishes containing:

- a) Grohmann agar, 1%  $\text{KNO}_3$  and a drop of yeast autolysate,
- b) 1% peptone agar, 1%  $\text{KNO}_3$ , 1% glucose, were inoculated.

All cultures were inoculated for 48 hours at 30° C. in a hydrogen atmosphere. With each of the two batches manometric experiments were made as described in Section 2. It appeared that the bacteria grown on Grohmann agar actively absorbed large amounts of hydrogen, whilst the bacteria grown on the rich organic medium did not show any absorption.

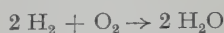
We must conclude from these experiments that production of hydrogenase is more or less completely suppressed, if the bacterium can rely on organic compounds as source of active hydrogen.

It is well known that denitrifying bacteria are able to dehydrogenate various organic substrates either with nitrate, or with free oxygen as a hydrogen acceptor. It seemed, therefore, interesting to investigate whether the same rule would hold in the case that molecular hydrogen acts as hydrogen donator. In other words the question should be answered whether "resting cells" of *M. denitrificans* able to reduce nitrate with hydrogen, are also capable of catalysing the so called "Knallgas"-reaction.

In an experiment in which again the manometric technique was employed this proved, indeed, to be the case. The results are given in Fig. 2.



From Fig. 2 it is clear that *M. denitrificans* is able to consume hydrogen and oxygen, as well as it is able to reduce nitrate with hydrogen. It should be realized that in the first case, according to the equation:



the hydrogen consumed is only two thirds of the total gas consumption, whilst in the second case the hydrogen consumption amounts to five fourths of the gas reduction observed.

The net hydrogen consumptions in the two cases are indicated in the figure by the dotted lines.

It is clear that in both conversions this consumption is practically the same, strongly suggesting that the capacity of the hydrogenase is in both cases controlling the rate of the reaction.

From these observations it can be concluded that the heterotrophic denitrifying bacterium *M. denitrificans* produces under certain cultural conditions a hydrogenase which enables it to use molecular hydrogen as a hydrogen donator. The hydrogen can then be transferred either to nitrate

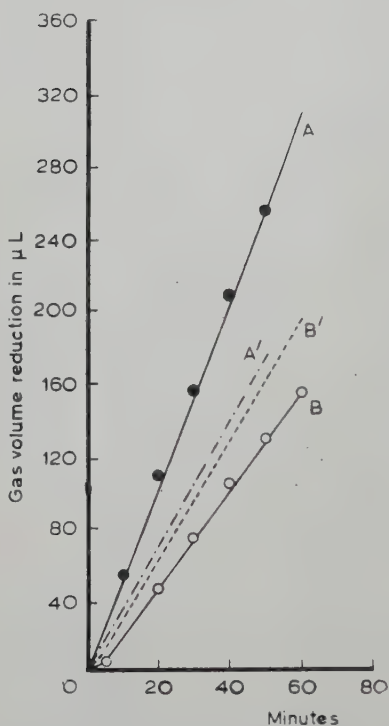


Fig. 2. *Micrococcus denitrificans* grown on Grohmann medium with nitrate and a drop of yeast autolysate in a hydrogen atmosphere.

Curve A, gas consumption in 50%  $\text{H}_2$ , 10%  $\text{O}_2$  and 40%  $\text{N}_2$  in the absence of nitrate. Curve B, gas reduction in hydrogen with nitrate. Curves  $A'$  and  $B'$  give the net hydrogen consumption in the two cases.

which is reduced to molecular nitrogen, or to free oxygen.

The latter implies that the bacterium belongs to the physiological group of the "Knallgasbakterien". To a certain extent the heterotrophic bacterium in question can be considered as a facultative autotrophe, although under these conditions evidently certain organic growth substances are essential, so that Lwoff's designation chemo-metatrophic seems more appropriate.

The fact that none of the other denitrifying bacterial species mentioned in Section 2 did show any development or hydrogen

absorption when inoculated into the mineral nitrate medium with yeast autolysate throws a clear light on the exceptional character of *M. denitrificans*.

### Summary.

"Resting cells" of pure cultures of various denitrifying bacteria which were grown on 1% peptone agar, 1% KNO<sub>3</sub>, 1% glucose in a hydrogen atmosphere were found to be unable to catalyse the reaction:



Enrichment cultures in a mineral nitrate containing medium in which molecular hydrogen was used as sole hydrogen donor invariably showed a large hydrogen consumption. Transfers, however, only maintained their activity, if some drops of yeast autolysate had been added to the fresh medium.

It was possible to isolate out of the transfers of these enrichment cultures a heterotrophic bacterium with a facultative chemotrophic mode of life in the sense of LWOFF.

An examination of the properties of the isolates showed that they possessed all the characteristics of *Micrococcus denitrificans*, as briefly described by BEIJERINCK, and in more detail by ELEMA and by SIJDERIUS. Moreover, the comparison with the 40 years old type strain of BEIJERINCK confirmed the identity of the isolates with this bacterium. A description of the general morphological and physiological characteristics of the species has been given.

*M. denitrificans* behaves in many cases as a normal heterotrophic denitrifying bacterium, but under appropriate cultural conditions the bacterium is able to grow with molecular hydrogen as hydrogen donor, using either nitrate or free oxygen as a hydrogen acceptor.

### References.

- BEIJERINCK, M. W. mit Mitwirkung von MINKMAN. D. C. J. 1910. Centralbl. f. Bakt. II, **25**, 30.  
ELEMA, B. 1932. De bepaling van de oxydatie-reductie potentiaal in bacteriëncultures en hare betekenis voor de stofwisseling. Thesis, Delft.  
GROHMANN, H. 1924. Centralbl. f. Bakt. II, **61**, 256.  
HOF, T. 1935. Rec. Trav. bot. néerl. **32**, 92.  
KASERER, H. 1906. Centralbl. f. Bakt. II, **16**, 681.

- KLUYVER, A. J. and MANTEN, A. 1942. *Antonie van Leeuwenhoek* **8**, 71.
- KLUYVER, A. J. 1953. Some aspects of nitrate reduction. In: Symposium Microbial Metabolism, Roma.
- KLUYVER, A. J. and VERHOEVEN, W. 1954. *Antonie van Leeuwenhoek* **20**, 241.
- LEBEDEFF, A. F. 1909. *Ber. deutsch. bot. Ges.* **27**, 598.
- LEWIS, D. 1951. *Biochem. J.* **49**, 149.
- LWOFF, A. 1943. *L'Évolution physiologique. Étude des pertes de fonctions chez les microorganismes.* Paris.
- NIKLEWSKI, B. 1914. *Centralbl. f. Bakt. II*, **40**, 430.
- VAN OLDEN, E. 1940. *Proc. Kon. Ned. Ak. v. Wet.* **43**, 635.
- ROBINSON, J. and GIBBONS, N. E. 1952. *Can. J. Botany* **30**, 147.
- RUHLAND, W. 1924. *Jahrb. f. wiss. Bot.* **63**, 321.
- SCHATZ, A. and BOVELL JR., C. 1952. *J. Bact.* **63**, 87.
- SCHLEGEL, H. G. 1953. *Arch. Mikrobiol.* **18**, 362.
- SÖHNGEN, N. L. 1906. *Het ontstaan en verdwijnen van waterstof en methaan onder de invloed van het organische leven.* Thesis, Delft.
- STEPHENSON, M. and STICKLAND, L. H. 1931 *a.* *Biochem. J.* **25**, 205.
- STEPHENSON, M. and STICKLAND, L. H. 1931 *b.* *Biochem. J.* **25**, 215.
- SIJDERIUS, R. 1946. *Heterotrophe bacteriën, die thiosulfaat oxyderen.* Thesis, Amsterdam.
- VERHOEVEN, W. 1952. *Aerobic sporeforming nitrate reducing bacteria.* Thesis, Delft.
- VERHOEVEN, W. and GOOS, J. J. C. 1954. *Antonie van Leeuwenhoek* **20**, 93.
- WOODS, D. D. 1938. *Biochem. J.* **32**, 2000.
-

(Research Department of N.V. Koninklijke Pharmaceutische Fabrieken v/h Brocades-Stheeman & Pharmacia, Amsterdam; University Hospital, Department for Internal Diseases, Leyden; Chemical Laboratory of the "Vrije Universiteit", Amsterdam).

## INVESTIGATION INTO THE TUBERCULOSTATIC ACTIVITY OF SOME DERIVATIVES OF p. AMINO-BENZOIC ACID<sup>1)</sup>

by

**C. VAN DER STELT, A. J. ZWART VOORSPUIJ  
and W. TH. NAUTA**

(Received November 12, 1953).

In 1940 WOODS made the important observation that p. amino-benzoic acid (PABA) abolished the bacteriostatic activity of the sulphonamides. The fact, so important for the explanation of the mechanism of action of the sulphonamides, was used by WOODS (39) and FILDES (19) as a starting point for their well-known theory. They contended that PABA has a very important function in the synthesis of products essential for the development of the micro-organism. Due to the structural relationship of the sulphonamides and PABA, a mutually competing interaction might be possible between these substances. Since the launching of this idea — which gave the chemist such useful indications in regard to the preparation of possible chemotherapeutics — many facts have emerged supporting it.

The size of this article does not allow of mentioning or discussing these facts. We confine ourselves to the remark that, also thanks to the method of "inhibition analysis" [initiated by SHIVE (37) and further developed by DE HAAN (24)], it is now possible to form a fairly satisfactory idea of the mechanism of action of the sulphonamides and of the rôle of PABA in bacterial metabolism. According to these authors, the sulphonamides prevent the for-

---

<sup>1)</sup> This research was supported in part by Riker Laboratories Inc. Los Angeles, California.

mation of a pteroylglutamic acid derivative of PABA which, when linked to a number of proteins, is responsible for various reactions — with introduction of a C atom — in the bacterial cell.

Our conceptions regarding the rôle of PABA in the metabolism of tubercle bacteria are far less detailed. *Mycobacterium tuberculosis* proves to belong to the group of micro-organisms able to synthesize PABA themselves (4, 16, 33). LEHMANN (28) failed to demonstrate a growth-stimulating effect in concentrations of  $10^{-3}$  to  $10^{-12}$  mol, although the literature also mentions contrasting observations (27). According to POPE and SMITH (34), PABA even acts as an "essential metabolite" in tubercle bacteria, as relatively small amounts of amino acid can abolish the inhibiting effect of sulphonamides and sulphones.

The tuberculostatic effect of p.aminosalicylic acid (PAS) proves also to be neutralized by the addition of PABA. In this case, however, an equivalent amount of the latter substance is required (26, 28). It is remarkable that PAS, although an antagonist of PABA, is active only against tubercle bacteria and not against other micro-organisms. According to TOBIE and JONES (38), this can probably be explained by the fact that PAS is able to replace PABA as a growth-stimulating substance for the majority of bacteria, which they proved for two mutations of *E. coli* strains.

It might transpire from the above facts that tubercle bacteria possess more than one chain of reactions for the synthesis of essential compounds, in which PABA is concerned. This would also follow from the absence of cross resistance between sulphonamide and PAS-resistant strains of *M. tuberculosis* (40).

Pteroylglutamic acid, of which PABA is a component, also has no growth-stimulating action in concentrations of  $10^{-3}$  to  $10^{-12}$  mol (28). POPE and SMITH (33) proved that this substance is also synthesized by the bacteria themselves. The latter is even unable to develop in higher concentrations of PABA. Various authors (20, 5, 36, 42) demonstrated this in vitro; mention is indeed made of a favourable influence on the survival time of infected guinea-pigs (6, 20, 21).

Although the rôle of PABA in the organism of tubercle bacteria is by no means clear, it is certain that it is significant. It therefore seemed fully justified to institute a detailed investigation into the effect of PABA derivatives on the growth of *M. tuberculosis*.

There is already a fairly extensive literature on the action of



these derivatives on micro-organisms other than tubercle bacteria, but so far little has been published on their tuberculostatic activity. A number of authors (5, 7, 17, 32) studied the activity of some PABA esters. BRUBACHER (7) also made active compounds by the preparation of isosters of PABA and their ethyl esters, although the effect of the most active substance, 2-(p. aminophenyl)-propene, was considerably reduced by the addition of serum.

BIOCCA (3) prepared a strongly active derivative by introducing the trichloroacetyl group into the amino group.

Study was also made of the tuberculostatic activity of a number of nucleus-substituted derivatives. Methyl substitution at the 2- or the 2,6-position only leads to inactive derivatives (18, 25). Introduction of a hydroxyl group in the ortho position to the carboxyl group, on the contrary, gives rise to the formation of the highly active p. aminosalicyclic acid, which holds such an important place in the chemotherapy of tuberculosis. This action, however, is rather specific. Substitution of the hydroxyl group by the methoxy group yields inactive derivatives (13, 15, 25), while substitution by a mercapto (13) or amino group (18, 23, 25) also abolishes the activity.

LEHMANN (28, 29) found that the corresponding derivative in which the hydroxyl group takes the meta position to the carboxyl group, was inactive. GHEORGHIU *et al.* (22) demonstrated great activity of PABA derivatives with a halogen atom in the ortho position to the carboxyl group. This activity would be equal to or would even exceed that of PAS. BERSCH and DÖPP (2), on the other hand, reported far lower activities. YOUMANS, DOUB and YOUMANS reported the tuberculostatic activities of 3500 organic compounds in the 4th review of the Chemical-Biological Coordination Center (41). Among these there are also some fifty compounds which may be considered as PABA derivatives. All these substances, however, are inactive (in concentrations  $< 100 \mu\text{g/ml}$ ) when tested on a medium to which serum has been added. The following substances belong to the simpler derivatives:

3,4-diamino-benzoic acid and its ethyl ester; 3,5-iodo-4-amino-benzoic acid ethyl ester; 3,5-diiodo-4-amino-benzoic acid ethyl ester; 4-amino-phthalic acid dimethyl ester; 4-amino-phthalic acid diethyl ester; 4-methyl-amino-benzoic acid; 4-dimethylamino-benzoic acid methyl ester; 4-acetyl-amino-benzoic acid ethyl ester; 4-nitrobenzoylamino-benzoic acid; 4-(methylsulphonamido)-benzoic acid; 4-guanidino-benzoic acid; 4-amino-hippuric acid; 4-amino-benzoylurea; 4-benzoylamino-acetophenone.

Esters of 4-nitro-benzoic acid were also involved in our investigation because, according to MILLER (30), this substance must also be considered as an antagonist of PABA. BUU HOÏ and CAGNIANT (8) had already observed a marked effect on tubercle bacteria of the hydnocarpyl and chaulmoogryl esters of 4-nitro-benzoic acid.

We tried to extend the number of data on the tuberculostatic activity of PABA derivatives by studying the following substances. When not stated otherwise, the compounds were prepared by the methods described in the literature.

#### EXPERIMENTAL PART.

Determination of the tuberculostatic activity *in vitro* was carried out on Loewenstein medium as modified by BEEUWKES (1) and on Youmans medium.

Columns I and II of the following tables show the concentration in  $\gamma$  per ml, at which there was complete inhibition of growth in the media of Beeuwkes and Youmans, respectively, with *M. tuberculosis* H37Rv, while columns III and IV give the results with a random strain isolated from a patient (P) or with the Ravemel strain (Ra), again in the media of Beeuwkes and Youmans, respectively.

##### A. Derivatives of 4-nitro-benzoic acid

| BS  |                                     | Activity |       |      |     | Literature |
|-----|-------------------------------------|----------|-------|------|-----|------------|
|     |                                     | I        | II    | III  | IV  |            |
| 726 | 4-nitro-benzoic acid                | 5        | > 8   | > 8  | 8   | (41)       |
|     |                                     |          |       | P    | P   |            |
| 727 | methyl-4-nitro-benzoate             | 5        | > 8   | 8    | > 8 |            |
|     |                                     |          |       | P    | P   |            |
| 728 | ethyl-4-nitro-benzoate              | 2        | 5     | 5    | > 8 |            |
|     |                                     |          |       | P    | P   |            |
| 729 | n-propyl-4-nitro-benzoate           | > 8      | 5     | > 8  | > 8 |            |
|     |                                     |          |       | P    | P   |            |
| 730 | i-propyl-4-nitro-benzoate           | 8        | > 8   | > 8  | > 8 |            |
|     |                                     |          |       | P    | P   |            |
| 741 | cyclopentyl-4-nitro-benzoate        | > 25     | —     | > 25 | 8   |            |
|     |                                     |          |       | P    | P   |            |
| 745 | 1,3-dimethyl-butyl-4-nitro-benzoate | 25       | 8     | 25   | 8   |            |
|     |                                     |          |       | P    | P   |            |
| 746 | 2-ethyl-butyl-4-nitro-benzoate      | 25       | 5     | > 25 | 25  |            |
|     |                                     |          | (irr) | Ra   | Ra  |            |

| BS   |                                                     | Activity |      |             |                   | Literature |
|------|-----------------------------------------------------|----------|------|-------------|-------------------|------------|
|      |                                                     | I        | II   | III         | IV                |            |
| 743  | 1-methyl-hexyl-4-nitro-benzoate                     | 25       | 8    | 25<br>Ra    | 8<br>Ra           | (9,31)     |
| 744  | 2-ethyl-hexyl-4-nitro-benzoate                      | 25       | 25   | 25<br>Ra    | 8<br>Ra           |            |
| 740  | 2,6-dimethyl-4-heptyl-4-nitro-benzoate              | > 25     | 0.5  | > 25<br>P   | 25<br>P           |            |
| 742  | 3,5,5-trimethyl-hexyl-4-nitro-benzoate              | 25       | 5    | > 25<br>P   | 5<br>P            |            |
| 750  | 3,5,5-trimethyl-cyclohexyl-4-nitro-benzoate         | 25       | 2    | > 25<br>P   | 5<br>P            |            |
| 1001 | 4-nitro-benzoic acid hydrazide                      | > 25     | > 25 | > 25<br>Ra  | > 25<br>Ra        |            |
| 1160 | 3-methyl-4-nitro-benzoic acid                       | > 100    | 25   | > 100<br>Ra | > 100<br>Ra       |            |
| 1186 | 4-nitro-isophthalic acid                            | > 100    | 1)   | > 100<br>Ra | > 100<br>Ra       |            |
| 747  | 4-nitro-phenyl-acetic acid                          | 25       | 25   | 25<br>Ra    | 25<br>Ra          |            |
| 748  | methyl-4-nitro-phenyl-acetate                       | 25       | 8    | 25<br>Ra    | 25<br>Ra          |            |
| 749  | ethyl-4-nitro-phenyl-acetate                        | 25       | 25   | 25<br>Ra    | 25<br>Ra<br>(irr) |            |
| 1101 | ethyl ester of 5-nitro-2-furoic acid                | 100      | 1    | 100<br>Ra   | > 100<br>Ra       |            |
| 1269 | methyl ester of 5-nitro-2-thiophene carboxylic acid | 100      | 25   | > 100<br>Ra | 25<br>Ra          |            |

1) no growth.

## B. 3 and 3,5-substituted 4-amino-benzoic acid derivatives.

| BS   |                                      | Activity |       |             |             | Literature |
|------|--------------------------------------|----------|-------|-------------|-------------|------------|
|      |                                      | I        | II    | III         | IV          |            |
| 1189 | 4-amino-3-methyl-benzoic acid        | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |            |
| 6286 | 4-amino-3,5-dimethyl-benzoic acid    | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |            |
| 1097 | 4-amino-3-chloro-benzoic acid        | > 100    | > 100 | 100<br>Ra   | 100<br>Ra   |            |
| 1098 | 4-acetyl-amino-3-chloro-benzoic acid | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |            |

| BS   |                                      | Activity |       |             |             | Literature   |
|------|--------------------------------------|----------|-------|-------------|-------------|--------------|
|      |                                      | I        | II    | III         | IV          |              |
| 6288 | methyl-4-amino-3,5-dichloro-benzoate | > 100    | > 100 | > 100<br>Ra | 100<br>Ra   |              |
| 1099 | 4-amino-3-bromo-benzoic acid         | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |              |
| 6289 | 4-amino-3,5-dibromo-benzoic acid     | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |              |
| 1078 | 4-amino-3-iodo-benzoic acid          | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |              |
| 1141 | 4-amino-3-nitro-benzoic acid         | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |              |
| 1136 | 4-acetylamino-3-nitro-benzoic acid   | > 100    | 25    | > 100<br>Ra | > 100<br>Ra |              |
| 1190 | 4-acetylamino-3-amino-benzoic acid   | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |              |
| 1187 | 4-amino-isophthalic acid             | > 100    | 1)    | > 100<br>Ra | > 100<br>Ra | 1) no growth |

## C. 2 and 2,6-substituted 4-amino-benzoic acid derivatives.

| BS   |                                      | Activity |       |             |             | Literature |
|------|--------------------------------------|----------|-------|-------------|-------------|------------|
|      |                                      | I        | II    | III         | IV          |            |
| 786  | 4-amino-2-methyl-benzoic acid        | > 8      | > 8   | > 8<br>P    | > 8<br>P    | (25,41)    |
| 6287 | 4-amino-2,6-dimethyl-benzoic acid    | > 100    | 25    | > 100<br>Ra | 100<br>Ra   | (18,25)    |
| 889  | 4-amino-2-chloro-benzoic acid        | 25       | 8     | 25<br>Ra    | 25<br>Ra    | (2,22)     |
| 1096 | 4-amino-2-bromo-benzoic acid         | > 25     | > 100 | 100<br>Ra   | > 100<br>Ra | (2,22)     |
| 1094 | 4-amino-2-iodo-benzoic acid          | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra | (22)       |
| 1095 | 4-acetylamino-2-iodo-benzoic acid    | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |            |
| 1088 | 2,4-diamino-benzoic acid             | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra | (18,23)    |
| 587  | 4-amino-2-hydroxy-benzoic acid       | 0.5      | 0.25  | 2           | 1           |            |
| 630  | 4-amino-2-mercapto-benzoic acid      | > 8      | > 250 | > 8<br>P    | > 250<br>P  | (13)       |
| 1159 | 4-amino-phthalic acid                | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra | (41)       |
| 632  | 3,3'-diamino-5,5'-dicarboxy-diphenyl | > 25     | > 25  | > 25<br>P   | > 25<br>P   |            |

## D. Derivatives of p. amino-benzoic acid in which the carboxyl group is modified.

| BS   |                                 | Activity |      |                    |             | Literature  |
|------|---------------------------------|----------|------|--------------------|-------------|-------------|
|      |                                 | I        | II   | III                | IV          |             |
| 1126 | methyl-4-amino-benzoate         | > 100    | 8    | > 100<br>Ra        | 25<br>Ra    | (5, 41)     |
| 1132 | n-propyl-4-amino-benzoate       | > 100    | 0.5  | > 100<br>Ra        | 25<br>Ra    | (5)         |
| 1039 | 4-amino-benzoic acid hydrazide  | > 100    | 25   | 100<br>(irr)<br>Ra | 1<br>Ra     | (9, 11, 31) |
| 698  | 4,4'-diamino-benzil             | —        | 25   | —                  | 25<br>P     | (41)        |
| 697  | 4,4'-diacetylamino-benzil       | —        | > 25 | —                  | > 25<br>P   | (41)        |
| 696  | 4,4'-diacetylamino-benzoine     | 25       | > 25 | 25<br>P            | (> 25)<br>P |             |
| 733  | 4,4'-diamino-benzophenone       | > 8      | > 8  | > 8<br>P           | > 8<br>P    |             |
| 732  | 4,4'-diacetylamino-benzophenone | > 8      | > 8  | —<br>P             | —<br>P      |             |
| 734  | 4,4'-diamino-diphenyl-methane   | —        | > 8  | 8<br>P             | 8<br>P      | (41)        |
| 700  | 4-acetylamino-benzaldehyd       | —        | > 25 | —                  | > 25<br>P   |             |

## E. Derivatives of p. amino-benzoic acid in which the amino group is modified.

| BS   |                              | Activity |              |             |             | Literature |
|------|------------------------------|----------|--------------|-------------|-------------|------------|
|      |                              | I        | II           | III         | IV          |            |
| 735  | 4-dimethylamino-benzoic acid | 8        | > 8          | —           | > 8<br>P    | (41)       |
| 756  | 4-butylamino-benzoic acid    | > 8      | (5)<br>(irr) | > 8<br>P    | irr<br>P    |            |
| 1133 | 4-n-hexylamino-benzoic acid  | > 100    | 8            | > 100<br>Ra | 8<br>Ra     |            |
| 1043 | 4-benzylamino-benzoic acid   | > 100    | contaminated |             | 50<br>Ra    |            |
| 1041 | 4-benzalamino-benzoic acid   | > 50     | > 100        | —           | > 100<br>Ra |            |
| 1040 | 4-acetylamino-benzoic acid   | > 100    | > 100        | > 100<br>Ra | > 100<br>Ra |            |



| BS   |                                        | Activity |       |             |             | Literature |
|------|----------------------------------------|----------|-------|-------------|-------------|------------|
|      |                                        | I        | II    | III         | IV          |            |
| 1033 | 4-benzoylamino-benzoic acid            | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra | (41)       |
| 758  | 4-mono-chloro-acetylamino-benzoic acid | > 8      | > 8   | > 8<br>P    | —<br>P      |            |
| 759  | 4-dichloro-acetylamino-benzoic acid    | > 8      | > 8   | > 8<br>P    | —<br>P      |            |
| 1127 | 4-carbethoxy-amino-benzoic acid        | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |            |
| 1134 | 4-ureido-benzoic acid                  | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |            |
| 1125 | 4-thioureido-benzoic acid              | > 100    | 25    | > 100<br>Ra | 100<br>Ra   |            |
| 1137 | 4-(benzoyl-thioureido)-benzoic acid    | > 100    | 8     | > 100<br>Ra | > 100<br>Ra |            |
| 1036 | 4-hydrazino-benzoic acid               | > 100    | > 100 | 100<br>Ra   | > 100<br>Ra |            |
| 736  | 4-nitroso-methylamino-benzoic acid     | 25       | 5     | 25<br>P     | 25<br>P     |            |

## F. Homologues and analogues of p. amino-benzoic acid.

| BS   |                                                  | Activity |       |             |             | Literature |
|------|--------------------------------------------------|----------|-------|-------------|-------------|------------|
|      |                                                  | I        | II    | III         | IV          |            |
| 760  | 4-amino-phenyl-acetic acid                       | > 8      | 8     | > 8<br>P    | > 8<br>P    | (2)        |
| 761  | ethyl-4-amino-phenyl-acetate                     | > 8      | > 8   | > 8<br>P    | > 8<br>P    |            |
| 710  | hexahydro-benzoic acid lactam                    | > 8      | > 8   | —           | —           |            |
| 699  | 2-amino-5-carboxy-pyridine                       | —        | > 25  | —           | 25<br>P     |            |
| 1191 | ethyl-ester of 5-amino-2-furoic acid             | 8        | 8     | 25<br>Ra    | > 100<br>Ra |            |
| 1270 | ethyl-ester of 5-amino-2-thiazol carboxylic acid | > 100    | > 100 | > 100<br>Ra | > 100<br>Ra |            |

Some of the substances investigated have not previously been reported in the literature. Their synthesis is therefore described below.

**4-n-hexylamino-benzoic acid.**

A mixture of 17.0 g 4-amino-benzoic acid, 7.0 g potassium hydroxide, 11.0 ml water and 20.0 g n-hexyl-bromide is heated under reflux for five hours. Subsequently the solid substance is separated from the reaction mixture, elutriated with plenty of water and crystallized from diluted alcohol or petroleum ether. The melting point is 120—121° C.

Yield: 15.0 g.

Analysis for  $C_{13}H_{19}NO_2$ : found 6.20%, calculated 6.33 %.

**4-thioureido-benzoic acid.**

This compound is twice mentioned in the literature. CASSON and PYMAN (10) prepared it from 4-amino-benzoic acid and potassium rhodanide; they obtained a product with a melting point higher than 330° C. DOUGLAS and DAINS (14) synthesized it by hydrolysis of the benzoyl compound, and found 255° C. as melting point. In order to study this controversy more closely, the thiourea compound was prepared by both methods.

The two substances thus obtained did not melt at temperatures lower than 330° C., however.

**3-amino-4-acetyl-amino-benzoic acid.**

280.0 g stannous chloride are dissolved in 540 ml glacial acetic acid by bubbling through gaseous hydrogen chloride. Subsequently 56.0 g of 4-acetyl-amino-3-nitro-benzoic acid are added gradually to this solution. After standing overnight the precipitate is filtered off. The solid substance is dissolved in water, after which hydrogen sulphide is led through in order to remove the tin salts. The stannous sulphide is filtered off and thrice boiled with alcohol. The desired compound is obtained by evaporation of the alcoholic solution; it can be purified further by dissolving in sodium carbonate solution, decolouration with carbon and precipitation with acid. Yield 25.0 g. Melting point 302° C. Molecular weight according to titration with baryta water: 191 (theoretical 194).

Analysis for  $C_9H_{10}O_3N_2$ : found 14.0; calculated 14.4% N.

The compound is only soluble readily in organic solvents (such as ethyl alcohol) when freshly prepared; its solubility is afterwards very poor.

**5,5'-dinitrodiphenic acid-2,2'.**

This acid was obtained from 2-amino-4-nitro-benzoic acid by the following method — not yet described in the literature — in an effort to prepare 2-chloro-4-nitro-benzoic acid.

2.0 g 2-amino-4-nitro-benzoic acid is dissolved in 7.0 ml 2 N sodium hydroxide. 0.66 g sodium nitrite is added to this solution, which is then filtered and slowly dripped into 28.0 ml 2 N hydrochloric acid cooled in ice. The diazonium solution prepared in this way is added to a solution of cuprous chloride in concentrated hydrochloric acid heated to 40° C. When there is no longer any development of nitrogen the mixture is heated for another five minutes at 60° C.; the solid substance is then filtered off and purified by solution in dilute ammonia, treatment of the solution with

carbon, and precipitation with dilute hydrochloric acid. Melting point 284–286° C. Yield 1.0 g.

The substance can easily be converted into the amino compound by boiling with a sodium sulphide solution (35).

#### Esters of 4-nitro-benzoic acid.

The esters were prepared according to the following general formula: 1 mole acid, 3 moles alcohol, 300 ml dichlorethane and 15.4 ml concentrated sulphuric acid are refluxed for fifteen hours (12). The reaction mixture is shaken with a sodium carbonate solution, the solvent removed and the residue distilled in vacuo. The table contains the data of the various esters.

| 4-nitro-benzoic acid esterified with | yield of ester from 10.0 g acid | melting point of ester in °C. | boiling point of ester in °C. | nitrogen content calculated in % | nitrogen content found in % |
|--------------------------------------|---------------------------------|-------------------------------|-------------------------------|----------------------------------|-----------------------------|
| 3,5,5-trimethyl-cyclohexanol         | 8.0                             | 77–78°                        | 190°/5 mm                     | 4.81                             | 4.81                        |
| 3,5,5-trimethyl-hexanol              | 15.0                            | 31–32°                        | 185°/5 mm                     | 4.78                             | 4.89                        |
| 2-ethyl-hexanol                      | 10.0                            | liquid                        | 175–180°/10 mm                | 4.68                             | 4.95                        |
| di-isobutyl-carbinol                 | 3.0                             | 46–50°                        | 210°/10 mm                    | 4.78                             | 4.71                        |
| cyclopentanol                        | 2.3                             | 55–58°                        | 150–155°/5 mm                 | 5.95                             | 5.85                        |
| 2-ethylbutanol                       | 10.0                            | liquid                        | 148–155°/5 mm                 | 5.57                             | 5.54                        |
| methyl-amyl-alcohol                  | 8.0                             | about 20°                     | 155°/5 mm                     | 5.57                             | 5.47                        |
| methyl-amyl-carbinol                 | 8.0                             | liquid                        | 150–155°/5 mm                 | 5.27                             | 5.28                        |

The following table gives some results of investigations as mentioned by other authors. We converted all their data in  $\gamma$ /ml.

| Name of the compound investigated | activity in $\gamma$ /ml       | medium according to                        | literature    |
|-----------------------------------|--------------------------------|--------------------------------------------|---------------|
| 4-nitro-benzoic acid              | $> 100 \gamma$                 | modified Proskauer and Beck with 10% serum | (41, No 2352) |
| 4-nitro-benzoic acid hydrazide    | $100 \gamma$<br>$< 100 \gamma$ | Dubois with Tween 80<br>Hohn               | ( 9)<br>(31)  |

| Name of the compound investigated | activity in $\gamma$ /ml                                                          | medium according to                                                               | literature               |
|-----------------------------------|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|--------------------------|
| methyl-4-amino-benzoate           | 1.9 $\gamma$<br>100 $\gamma$                                                      | ?<br>modified Proskauer and Beck with 10% serum                                   | (5)<br>(41, No 1794)     |
| n-propyl-4-amino-benzoate         | 0.09 $\gamma$                                                                     | ?                                                                                 | (5)                      |
| 4-amino-benzoic acid hydrazide    | partial inhibition at<br>100 $\gamma$<br>40 $\gamma$<br>only very slightly active | Dubos with Tween 80<br><br>Hohn<br>Proskauer and Beck with 10% serum              | ( 9)<br><br>(31)<br>(11) |
| 4-dimethylamino-benzoic acid      | 100 $\gamma$                                                                      | modified Proskauer and Beck with 10% serum                                        | (41, No 1780)            |
| 4-hydrazino-benzoic acid          | > 100 $\gamma$                                                                    | modified Proskauer and Beck with 10% serum                                        | (41, No 2515)            |
| 4-amino-2-methyl-benzoic acid     | 1500 $\gamma$<br>> 100 $\gamma$                                                   | Dubos with Tween 80<br>modified Proskauer and Beck with 10% serum                 | (25)<br>(41, No 1778)    |
| 4-amino-2,6-dimethyl-benzoic acid | 84 $\gamma$<br>167 $\gamma$<br><br>33 $\gamma$                                    | Dubois with Tween 80<br>Kirchner with 10% serum<br>Lockemann with surface culture | (25)<br>(18)<br>(18)     |
| 4-amino-2-chloro-benzoic acid     | as active as PAS<br>10 $\gamma$                                                   | ?<br>Sauton                                                                       | (22)<br>( 2)             |
| 4-amino-2-bromo-benzoic acid      | 4 $\times$ as active as PAS<br>20—50 $\gamma$                                     | ?<br>Sauton                                                                       | (22)<br>( 2)             |
| 4-amino-2-iodo-benzoic acid       | as active as PAS                                                                  | ?                                                                                 | (22)                     |

| Name of the compound investigated | activity in $\gamma$ /ml | medium according to                        | literature    |
|-----------------------------------|--------------------------|--------------------------------------------|---------------|
| 2,4-diamino-benzoic acid          | 125-250 $\gamma$         | modified Dubos                             | (23)          |
|                                   | 76 $\gamma$              | Dubos with 0.05% Tween 80                  | (18)          |
|                                   | > 152 $\gamma$           | Kirchner with 10% serum                    | (18)          |
|                                   | > 152 $\gamma$           | Lockemann with surface culture             | (18)          |
| 4-amino-2-mercapto-benzoic acid   | > 100 $\gamma$           | synthetic medium with 10% serum            | (13)          |
| 4-amino-phthalic acid             | > 100 $\gamma$           | modified Proskauer and Beck with 10% serum | (41, No 1852) |
| 4-amino-phenylacetic acid         | 20 $\gamma$              | Sauton                                     | ( 2)          |
| 4,4'-diamino-diphenyl-methane     | > 10.0 $\gamma$          | modified Proskauer and Beck with 10% serum | (41, No 2086) |
| 4,4'-diamino-benzil               | 100 $\gamma$             | id.                                        | (41, No 2170) |
| 4,4'-diacetylamino-benzil         | > 100 $\gamma$           | id.                                        | (41, No 2174) |

### Discussion.

Of all the substances investigated, only the nitro-benzoic acid derivatives showed occasional activity, especially on the Youmans medium. The activity was in general lost when they were investigated on the protein-containing Beeuwkes medium. This difference is also striking for the propyl ester of PABA. It was impossible to reproduce the high activities reported by GHEORGHIU *et al.* for PABA derivatives halogen-substituted in position 2.

As far as comparison is possible, the activities found by us in general do not deviate significantly from those mentioned by other authors.

### Summary.

Seventy-five compounds, derivatives of 4-nitro-benzoic acid and of 4-amino-benzoic acid, were investigated for their tuberculostatic



activity. The majority showed practically no activity in the concentrations studied. A number of 4-nitro-benzoic acid esters of branched alcohols, which proved to be active on the synthetic medium, showed a considerably lower activity on the protein-containing Beeuwkes medium.

The synthesis of the esters mentioned, and that of n-hexylamino-benzoic acid, 4-acetylamino-3-amino-benzoic acid, and 5,5'-dinitro-2,2'-dicarboxy-diphenyl is described. The melting point of 4-thio-ureido-benzoic acid was higher than 330° C. in our investigation, and was not at 255° C. as mentioned in the literature.

### L i t e r a t u r e.

1. BEEUWKES, H. 1950. Ned. Tijdschr. v. Geneesk. **94**, 3347; *Antonie van Leeuwenhoek* **16**, 359.
2. BERSCH, H. W. and DÖPP, W. 1951. *Arch. Pharm.* **284**, 30.
3. BIOCCA, E. 1932. *Arquiv. biol.* **29**, 97; ref. C. A. **40**, 1932.
4. BIRD, O. D. 1947. *Nature* **159**, 33.
5. BLOCH, H., BRUBACHER, H., ERLÉNMEYER, H. and SUTER, E. 1947. *Helv. Chim. Acta* **30**, 539; BLOCH, H., LEHR, H. and ERLÉNMEYER, H. 1945. *Helv. Chim. Acta* **28**, 1406.
6. BLOOMBERG, B. M. 1947. *S. African J. Med. Sci.* **12**, 1; ref. C. A. **42**, 2025.
7. BRUBACHER, G. 1950. *Systematische Untersuchungen über tuberkulostatisch wirksame primäre Amine*. Diss. Basel; BRUBACHER, G. and SUTER, E. 1950. *Helv. Chim. Acta* **33**, 256.
8. BUU-HOÏ, N. P. and CAGNIANT, P. 1944. *Naturwissenschaften* **32**, 83; BUU-HOÏ, N. P., CAGNIANT, P. and MENTZER, Ch. 1944. *Bull. Soc. Chim. France* **11**, 127.
9. BUU-HOÏ, N. P., WELSCH, M., DECHAMPS, G., LE BIHAN, H., BINONAND, F. and MENTZER, Ch. 1952. *Compt. rend.* **234**, 1925.
10. CASSON, A. P. T. and PYMAN, F. L. 1931. *J. Chem. Soc.* p. 2991.
11. CAVALLINI, G., MASSARANI, E., MAZZUCCHI, F. and RAVENNA, F. 1952. *Il Farmaco* **7**, 138.
12. CLINTON, R. O. and LASKOWSKI, S. C. 1948. *J. Am. Chem. Soc.* **70**, 3135.
13. DOUB, L., SCHAEFER, J. J., BAMBAS, L. L. and WALKER, C. T. 1951. *J. Am. Chem. Soc.* **73**, 903.
14. DOUGLAS, I. B. and DAINS, F. B. 1934. *J. Am. Chem. Soc.* **56**, 1408.
15. DRAIN, D. J., GOODACRE, C. L. and SEYMOUR, D. E. 1949. *J. Pharm. Pharmacol.* **1**, 784.
16. EKSTRAND, T. and SJÖGREN, B. 1945. *Nature* **156**, 476.
17. ERLÉNMEYER, H., NOLL, H. and ŠORKIN, E. 1949. *Helv. Chim. Acta* **32**, 605.
18. ERLÉNMEYER, H., PRIJS, B., ŠORKIN, E. and SUTER, E. 1948. *Helv. Chim. Acta* **31**, 988.
19. FILDES, P. 1940. *Lancet* **238**, 955.

20. DI FONZO, M. 1945. Boll. soc. ital. biol. sper. **20**, 832; ref. C. A. **40**, 6545.
  21. DI FONZO, M. 1947. Farm. Sci. e tec. (Pavia) **2**, 287; ref. C. A. **42**, 1625.
  22. GHEORGHIU, C. V., STOICESCU-CRIVETZ, L., TOMA A. and CONSTANTINESCU, N. 1949. Acad. Rep. Populare Române Bul. Stiint. Sect. A **1**, 993; ref. C. A. **45**, 7249.
  23. GOODACRE, C. L., MITCHELL, B. W. and SEYMOUR, D. E. 1948. Quart. J. Pharm. and Pharmacol. **21**, 292.
  24. DE HAAN, P. G. 1953. Het werkingsmechanisme van sulfanilamide. Diss. Utrecht; DE HAAN, P. G., WINKLER, K. C. and BALJET, H. 1949. Antonie van Leeuwenhoek **15**, 136; DE HAAN, P. G. and WINKLER, K. C. 1951. Antonie van Leeuwenhoek **17**, 107.
  25. HIRT, R. and HURNI, H. 1949. Helv. Chim. Acta **32**, 378.
  26. HURNI, H. 1949. Schweiz. Z. Path. u. Bakt. **12**, 282.
  27. LEGEŹYŃSKI, S. and SLOPEK, S. 1949. Med. Doświadczał i Mikrobiol. **1**, 611; ref. C. A. **45**, 6754.
  28. LEHMANN, J. 1949. Experientia **5**, 365.
  29. LEHMANN, J. 1946. Lancet **250**, 15; Svenska Läkartidn. **43**, 2029; ref. C. A. **41**, 1334.
  30. MILLER, J. K. 1941. J. Pharmacol. **71**, 14.
  31. OFFE, H. A., SIEFKEN, W. and DOMAGK, G. 1952. Naturwissenschaften **76**, 446.
  32. PISU, I. 1950. Farm. Sci. e. tec. **5**, 269; ref. C. A. **44**, 9509.
  33. POPE, H. and SMITH, D. T. 1946. Amer. Rev. Tuberc. **54**, 559.
  34. POPE, H. and SMITH, D. T. 1950. Amer. Rev. Tuberc. **62**, 34.
  35. PUFAHL, F. 1929. Ber. **62**, 2817.
  36. DI RAIMONDO, F. and ORTALI, N. 1948. Rend. ist. super. sanità **11**, 1230; ref. C.A. **43**, 6275.
  37. SHIVE, W. 1951. Vitamins and Hormones **9**, 76.
  38. TOBIE, W. C. and JONES, M.J. 1949. J. Bact. **67**, 573.
  39. WOODS, D. D. 1940. Brit. J. Exp. Pathol. **21**, 74; WOODS, D. D. and FILDES, P. 1940. Chem. Ind. **59**, 133.
  40. YEGIAN, D. and LONG, R. T. 1951. J. Bact. **61**, 747.
  41. YOUmans, G. P., DOUB, L. and YOUmans, A. S. 1953. The Bacteriostatic Activity of 3500 Organic Compounds for *Mycobacterium tuberculosis* var. *hominis*. Chemical Biological Coordination Center Review No 4.
  42. ZETTERBERG, B. 1949. Acta Pathol. Microbiol. Scand. Suppl. **82**, 1; ref. C.A. **43**, 7545.
-

(Centraal Bureau voor Schimmelcultures, Baarn, Holland).

## *ASPERGILLUS ASPERESCENS* N. SP.

by

AMELIA C. STOLK

(Received August 17, 1953).

Three strains of this *Aspergillus* have been examined. One strain was sent to the Centraal Bureau voor Schimmelcultures, Baarn, for identification in the summer of 1952. It was isolated by Mr. M. W. STARZECKI from dung of bats in the cave Nietoperzowa near Krakow, Poland. This fungus was not identical with any of the species described in THOM and RAPER's "A Manual of the *Aspergilli*" (4), yet it resembled in all respects two strains of an *Aspergillus*, sent to the C.B.S. in 1951 by Dr P. W. BRIAN under the name of *Aspergillus humicola* Chaudhuri et Sachar. Strain 385 was isolated in 1947 from Read's Cavern in Somerset and strain 517 was collected in 1948 from a deep cave at Ogof Flynnon Ddu, Brecon. In our opinion some characteristics of Dr BRIAN's fungi, especially the size and shape of the conidia, did not agree very well with the description of *A. humicola* by CHAUDHURI and SACHAR (1). We therefore sent Dr BRIAN's 385 and the polish strain to Dr K. B. RAPER, asking his opinion regarding these fungi. Dr RAPER, who very kindly examined the fungi, suggested that these strains should be described as a new and non-ascosporic species of the *A. nidulans* series.

### Macroscopical characters.

Colonies on Czapek's solution agar growing restrictedly, attaining a diameter of 2.5 cm in two weeks at room temperature; azonate, to slightly zonate; furrowed, with central area usually somewhat buckled; consisting of a fairly tough basal felt, bearing conidiophores mostly as branches from interlacing hyphae, becoming velvety or nearly so at the margin; light to medium sporing; at first white, passing through shades of citron green <sup>1)</sup>, olive-buff and olive-

---

<sup>1)</sup> Colours are indicated in terms of RIDGWAY (3).

ocher to course green and jade green, becoming dark olive-buff in age; exudate fairly abundant, in small drops, clear to pale yellow, reverse of colonies at first pale yellow to citrine-drab, after two weeks deep olive, tawny-olive or orange-cinnamon, with surrounding agar usually coloured in pale yellow-brown shades.

Colonies on malt agar growing faster than on Czapek agar, up to 4 cm in diameter in two weeks at room temperature; showing concentric zones; consisting of a basal felt with centre somewhat buckled and floccose, margin velvety or nearly so; often showing a tendency to develop colourless to yellow mycelial overgrowths; medium sporing; the broad white margin is passing through zones of course green, mignonette green and ecru-olive to a floccose centre in shades of serpentine green, isabella colour or buffy olive; exudate limited, clear; reverse of colonies zonate, in shades of chamois and lime green.

Colonies on cherry agar growing very restrictedly up to 2 cm in diameter in two weeks at room temperature; zonate; medium sporing; margin broad, white, zones in shades of dark olive-buff, light hellebore green and hellebore green; exudate limited, clear; reverse dull citrine.

Frequently the cultures are showing a tendency to produce overgrowths as colony sectors. Colonies in tubes may in relatively short time degenerate into flocculent and lightly sporulating forms. It is possible, however, to maintain the typical colony aspect by transferring only the conidia of the more velvety parts of the colonies.

The colours of the cultures may vary strongly, depending on the culture medium and on the amount of the produced overgrowths.

CURTIS *et al.* (2) reported the production of the antifungal substance humicolin by the strains Brian 385 and 517.

### Microscopical characters.

Conidial heads typically radiate, globose, commonly splitting into well defined columns in age; up to 150  $\mu$  in diameter, but sometimes reduced to small clusters of sterigmata. Conidiophores, when arising from the substratum, up to 600  $\mu$  in length by 5 to 9  $\mu$  in diameter, as branches from aerial hyphae, ranging in length from very short to 150  $\mu$  by 3.5 to 5  $\mu$  in diameter; with smooth walls, in young heads pale brown, in older heads cinnamon brown. Vesicles 9 to 15  $\mu$  in diameter, hemispherical to globose, fertile over almost the entire surface in larger heads, in smaller heads the fertile area is only covering the upper half of the vesicle (fig. 1a, 3 and 5); pale brown in

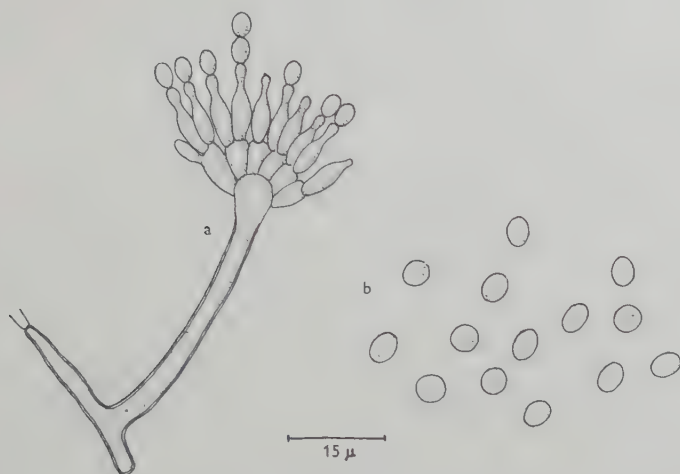


Fig. 1. *Aspergillus asperescens*.

*a.* young conidial head, mounted in lactic acid, culture 10 days old.

*b.* Smooth conidia of a 10 day old culture.



Fig. 2. *Aspergillus asperescens*.

*a—c.* Reduced conidial heads of a 10 day old culture.

*d.* Clusters of sterigmata along the conidiophore.



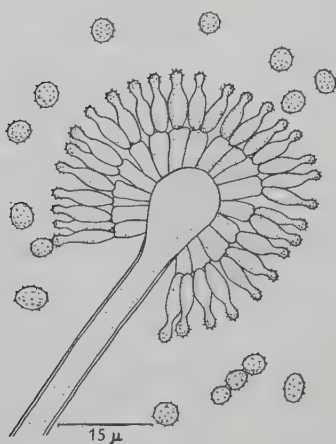


Fig. 3. *Aspergillus asperescens*. Large conidial head and rough conidia of a 3 week old culture.

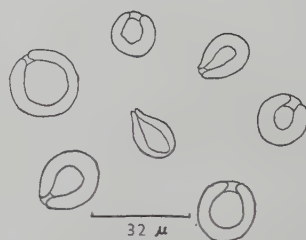


Fig. 4. *Aspergillus asperescens*. Hülle cells.

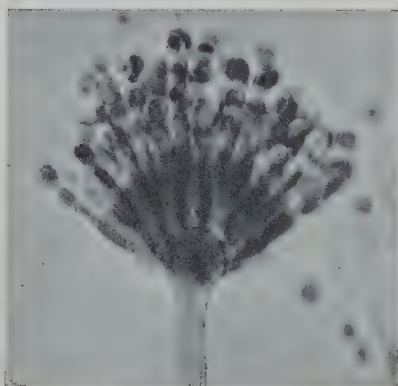


Fig. 5. *Aspergillus asperescens*. Young conidial head, mounted in lactic acid, coloured with cotton blue, culture 10 days old,  $\times 850$ .

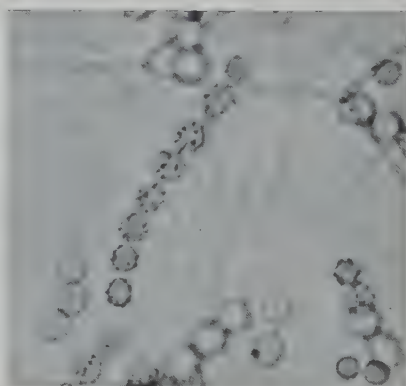


Fig. 6. *Aspergillus asperescens*. Rough conidia of a 3 week old culture,  $\times 850$ .

colour. Sterigmata in two series, primary commonly  $6$  to  $9\ \mu$  by  $3$  to  $3.5\ \mu$ , secondary  $7.5$  to  $9\ \mu$  by  $3$  to  $3.5\ \mu$ . Conidia in young cultures subglobose to elliptical, smooth,  $4$  to  $6\ \mu$  by  $3.5$  to  $4.5\ \mu$ , occasionally up to  $7\ \mu$  in long axis (fig. 1*b* and 5); in cultures older than two weeks the produced conidia are conspicuously roughened from co-

louring substance deposited as tubercles and spines between the primary and secondary walls, globose to subglobose, 4 to 5  $\mu$  by 3.5 to 4.5  $\mu$ , occasionally up to 6  $\mu$  (fig. 3 and 6), green coloured, borne in long radial chains. Globose to subglobose hülle cells, closely resembling those of *A. nidulans* are produced in central colony areas on maltagar, 15 to 24  $\mu$  by 14 to 18  $\mu$  (fig. 4). No sclerotia or perithecia have been found.

In all three examined strains, reduced conidial heads are formed, ranging from normal structures with short conidiophores to simple sterigmata along trailing aerial hyphae (fig. 2*a, b, c*). Sometimes clusters of sterigmata appear along the ascending conidiophores (fig. 2*d*).

In very young cultures only smooth conidia are found, older cultures show both smooth and rough conidia, whereas in cultures of more than a month old, mainly rough conidia are seen. Close observation of the development of conidial heads in monospore cultures, showed that at first only smooth conidia are produced (fig. 1*b* and 5). In cultures older than two weeks, rough conidia are formed, even the immature conidia, still attached to their sterigmata, may have a distinctly roughened wall (fig. 3).

The fungus differs from all other species of *Aspergillus* hitherto described. As Dr RAPER (in litt.) pointed out the brown conidiophore and the hülle cells place it in the *A. nidulans* group. The fungus, however, may represent a transition in the direction of the *A. versicolor* group, since the species of that group are likewise characterized by globose conidial heads. In many respects this fungus resembles *A. humicola* Chaudhuri et Sachar. Unfortunately no type material of this species exists, so it was only possible to compare our three strains with the description of that species by CHAUDHURI and SACHAR. Some important differences were proved. Our fungus has a brown conidiophore, hülle cells, and in age large, subglobose, rough conidia, 4 to 5  $\mu$  by 3.5 to 4.5  $\mu$ , whereas *A. humicola* has an almost colourless conidiophore and small, smooth, globose conidia 2 to 3  $\mu$  in diameter. It is better that this fungus be regarded as a new *Aspergillus* species. The fungus is named *A. asperescens* because of the character of the conidium wall.

*Aspergillus asperescens* sp. n.

Coloniae in agarō Czapeki lente crescentes; structura pilosa, in

medio paulum floccosa, ad marginem versus paulum speciem vellueti praebens; primo alba, deinde flavo-viridis; parte aversa rufo-fusca; in agarō multi zonae formantur. Capitula conidiofera rotunda, diametro usque ad  $150\ \mu$ ; saepe capitula reducta. Conidiophora a brevissimis usque ad  $600\ \mu$  longa,  $3,5 - 9\ \mu$  lata; pariete glabro, fusco. Vesicula rotunda,  $9 - 15\ \mu$ . Duo ordines sterigmatum in magnis capitulis totam vesiculam operiunt, in parvis capitulis tantum dimidiam partem superiorem; sterigmata primaria  $6 - 9\ \mu$  longa,  $3 - 3,5\ \mu$  lata, sterigmata secundaria  $7,5 - 9\ \mu$  longa,  $3 - 3,5\ \mu$  lata. Conidia in culturis recentibus ovis similia, glabra,  $4 - 6\ \mu$  longa,  $3,5 - 5\ \mu$  lata, in culturis veteribus asperissima,  $4 - 5\ \mu$  longa,  $3,5 - 4,5\ \mu$  lata, viridia. Cellulae "hülle" rotundae vel ovis similes, in agarō multi  $15 - 24\ \mu$  longae,  $14 - 18\ \mu$  latae. Nulla perithecia, nulla sclerotia.

Hab. In stercore vespertilionum in antro apud Cracoviam (Polonia); in solo in antro, cui nomen est Read's Cavern, Somerset (Britannia); in antro in Ogof Flynnon Ddu, Brecon (Britannia).

Type material, Dr BRIAN's strain 385, will be deposited at the Herbarium of the Royal Botanic Gardens, Kew and at the Rijks Herbarium, Leiden. Living cultures are maintained at the Commonwealth Mycological Institute, Kew, the Northern Regional Research Laboratory, Peoria and the Centraal Bureau voor Schimmelfcultures, Baarn.

### Summary.

A new *Aspergillus* species, *Aspergillus asperescens*, is described. Three strains of this fungus have been isolated from soil and dung of bats in caves in Great-Britain and Poland. It is characterized by a yellow green colour, radiate conidial heads, two series of sterigmata, a brown conidiophore, hülle cells and subglobose conidia, which are smooth in young and rough in old cultures.

### References.

- CHAUDHURI, H. and SACHAR, G. S. 1934. Ann. mycol. **32**, 90.  
CURTIS, P. J., HEMMING, H. G. and JEFFRYS, E. G. 1952. Trans. Brit. mycol. Soc. **35**, 263.  
RIDWAY, R. 1912. Color standards and color nomenclature, Washington, D.C.  
THOM, C. and RAPER, K. B. 1945. A manual of the Aspergilli, Baltimore.

(From the Department of Hygiene of the Laboratory of Hygiene of the State University, Utrecht).

## THE COUNTING OF VIABLE TUBERCLE BACTERIA

by

**R. VOORHORST**

(Received January 30, 1954).

Quantitative work with tubercle bacteria always has been based on principles different from those applicable to ordinary bacteria.

Weighing, practically never used in quantitative bacteriological work, has been the universally used measure for the clumpy material the tubercle bacteria will produce in culture media (CRANDALL, 1949). Counting may be realised by emulgating, filtering through a suitable filter and filling into a bacteria counting chamber. Relative counting in proportion to erythrocytes in smears of mixtures of known volumes of blood and suspension of bacteria is another classical method (WRIGHT, cf. JENNINGS, 1926). It has been proposed (YOUMANS, 1946) to estimate the mass of bacteria by micro Kjeldahl determination of nitrogen. Nephelometric methods can be used (WOLINSKY and STEENKEN, 1947; SMITH, 1947) if surface active substances, such as Tween 80, have induced homogeneous growth of the tubercle bacteria.

None of these techniques however allow differentiation between dead and viable bacteria; besides several of them ask for highly concentrated suspensions.

VANDIVIERE *et al.* (1952) estimated viable germs by colorimetric determination after acetone extraction of the red formazan which is formed in living bacteria — but not in dead — from 2.3.5 triphenyl-tetrazoliumchloride and found a satisfactory correlation with viable counts.

Surface counts can be realised by inoculation on solid media of known volumes of serial dilutions. Viable units per unit of volume can thus be determined fairly accurately, as recently has been demonstrated by the work of PANNISET and MARCIL (1953).

Accurate to a high degree appears to be the "surface drop counting method" as published by FENNER (1951). A number of drops of constant volume and a suitable content of viable units of tubercle bacteria are dropped separately on the surface of a well dried albumen-oleinic acid-agar plate (Dubos). Colonies are allowed to develop in 3-5 weeks of incubation at 37° C. and up to a number of 50 per drop can be counted under a colony microscope; the error would be limited to 20%. To combat contamination during the long period of incubation penicillin, combined with either grisein or actidion were added to the medium. The medium is uneconomically utilized which makes for a rather expensive method specially so by the high cost of Bovine albumen. The same holds true for the classical dish-counting method of YEGIAN and BUD (1951), in the Dubos albumen-oleinic acid medium. Per plate no more than one counting can be performed after 30-35 days of incubation. It asks for still more medium giving essentially the same results as the FENNER technique. Because the dishes have to be opened less frequently danger for contamination is considerably reduced. Finally CHRISTENSEN *et al.* (1953) described a counting method with the aid of the roller flask technique.

This invited us to describe our experiences, having worked along similar lines since 1951 for big scale viable counts of tubercle bacteria. As early as 1938 the principle of the "agar-plates rolled in flasks" was introduced and worked out by JULIUS (1938) in this laboratory.

From that time on it has been used in a number of researches and routine counts in the institute of its origin and elsewhere. The method has been under a procedure of modification and adaptation for the sake of big scale viable counts of tubercle bacteria in relation to investigations on the mode of action of chemotherapeutics and antibiotics (to be published).

The following adaptations and improvements especially devised for tubercle bacteria, have been introduced. For the essentials compare the original technique (JULIUS, 1938).

A. The medium used is a modification of one of the Dubos media as a result of systematic investigations by Mrs F. VOORHORST-SMEENK. (to be published). It is prepared from four stock solutions of the following composition.

Ia. Albumen-salt-mixture.  $\text{KH}_2\text{PO}_4$  1 g,  $\text{Na}_2\text{HPO}_4$  2.5 g,  $\text{ZnSO}_4$  0.1 mg,  $\text{CuSO}_4$  0.1 mg,  $\text{MgSO}_4$  7aq. 10 mg,  $\text{CaCl}_2$  0.5 mg, ca-



seinhydrolysate (bacto) 2 g, asparagine 2 g, NaCl 5 g, bovine albumen (Poviet) 25 g, aqua dist. 500 ml.

The pH has to be adjusted to 6.8, sterilisation is done by pressure filtration through an E.K.pad.

Ib.  $\text{FeNH}_4$  citrate 200 mg, aqua dist. 100 ml. Sterilization by means of Chamberland-candle filtration.

II. 497.5 ml of stock solution Ia plus 2.5 ml of solution Ib make 500 ml albumen-iron salt mixture.

III. Solution of phospholipides.

10 g of animal (spinal cord) phospholipides are dissolved in 100 ml boiling 96% ethanol. This is forced through a fine needle into 1000 ml of distilled water under vigorous mechanical stirring, then filtered through cottonwool on a Buchner-funnel, followed by sterilization for 1 hour at 100° C. This heating allows the alcohol to evaporate. A stable colloidal solution of phospholipides results consisting mainly of kephalines, sphingomyelins and cerebrosides.

IV. Water Agar.

Bacto-agar 3% in water is heated for 20 minutes at 100° C. After cooling to 60-70° C. it is filtered through cotton and filter-paper on a Buchner funnel and sterilized for 20 minutes at 115° C.

Preparing the medium for use is done by bringing all three components to 56° C. (no. IV in melted state) and adding to 25 ml of IV, 20 ml of II and 5 ml of III.

The final medium then will contain: agar 1.5%, bovine albumen 2%, phospholipides 0.1%, caseinhydrolysate 0.16%, asparagine 0.16%, NaCl 0.4%,  $\text{KH}_2\text{PO}_4$  0.08%,  $\text{Na}_2\text{HPO}_4$  0.2%,  $\text{ZnSO}_4$  0.00008%,  $\text{CuSO}_4$  0.00008%,  $\text{MgSO}_4$  0.008%,  $\text{CaCl}_2$  0.0004%,  $\text{FeNH}_4$  citraat 0.04%.

**B.** The protamin-zinc-insuline flask as originally used by JULIUS have disappeared from the market. To find suitable substitutes caused some considerable difficulties untill the modern standardised flask of 20 ml (fig. 1) came into general use. These are so well shaped that making new adaptors to the setting apparatus was accepted by all users of the technique in the institute. The flasks are perfect cylinders all equally wide, made of even, thin, but strong, clear glass and provided of good flat bottom. Being a massproduct they are not expensive. If, as in case of working with highly pathogenic germs, the neck will be too narrow, so that by quick working touching the rim provokes chances of infection and contamination, the collar

can be cut off and the neck can be widened in the flame by a standard cone.

**C.** The quantity of medium per flask will conveniently be of 2 ml. The agar concentration should not be under 1.5%. Filling the flask with agar may cause some difficulty as a consequence of the narrow temperature allowance. It should not surpass 56° C. nor come down below 45° C. The syringe with spring, being of too bulky a mass for smooth temperature adaptation, better can be substituted by a sterile pipet, preheated in a Bunsen flame. After filling the flasks are



Fig. 1.

stored in the water bath of 45° C. till the very moment of inoculation.

The flasks are plugged with cotton wool during all manipulations. After setting, in the roller apparatus the cotton plugs are replaced by small rubber stoppers (fig. 1).

**D.** As in the original version of the technique quantitative inoculation is done by drops from a Pasteur pipet cut at a standardised external diameter (JULIUS, 1938). Inoculations are always done in duplo.

**E.** It appeared that as a consequence of horizontal position of the axes of the setting apparatus it hardly could be avoided that small

quantities of the infected medium touched the cottonplug. This on the one hand might cause a small counting error, on the other hand however, which is much more important, it may be a serious danger of infection to the staff members handling virulent tubercle bacteria. By raising the number of rotations up to about 2000 r.p.m. centrifugal force will exceed so many times gravity that without any untoward consequence the axes may be brought to an inclination of  $15^\circ$  with the horizontal (fig. 2); it even may be tilted considerably more. In this way contamination of the cotton plugs is avoided with certainty. Care has to be taken though that the cotton will tightly plug the necks otherwise at this high rotating speed they may be thrown off the flasks.

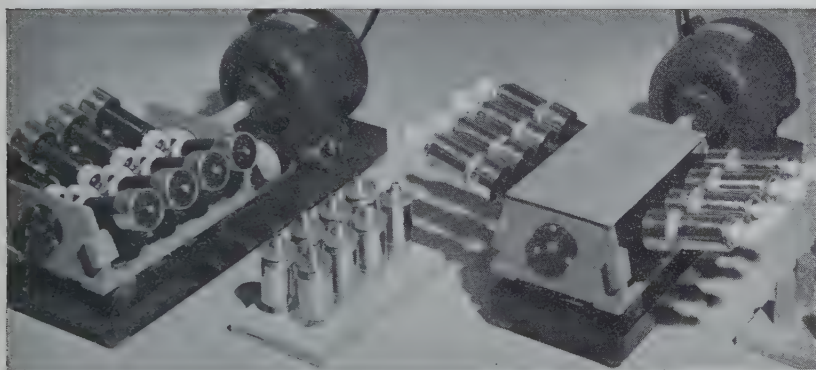


Fig. 2.

Perhaps contrary to expectations rotating the liquid agar in the setting machine will not warrant sufficient mixing with the inoculum. Test countings have given support to the originally observed but frequently neglected necessity to mix the contents of the flask thoroughly by hand after inoculation and before fixing them on the rotators. If on warm days setting of the agar is unduely delayed this may be overcome by precooling the metal adaptors in ice water. They will store enough cold to warrant rapid setting of the agar. When taking out the flasks the agar has to be checked for an exact and evenly setted layer against the wall of the flask. Incubation is done in vertical position bottom down.

**F.** In spite of the most meticulous care for sterile working we frequently saw a non-acceptable number of airborne contaminations.

So adding 5 Units of penicillin per ml to the medium must be recommended. It came out to be sufficient to reduce contaminations to a minimum without influence on the development of the colonies. In this medium, five days of incubation will yield countable colonies of our homogeneously growing strain D 328 (fig. 3). The incubation period for the H37Rv strain is 7-10 days. It may well be understood and underlined here that the problem of homogeneity of the inoculum is left apart for the time being.

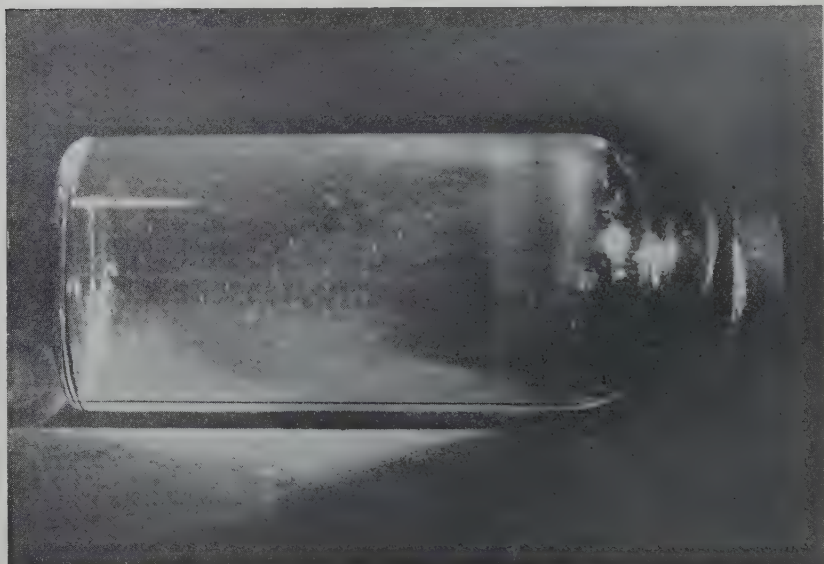


Fig. 3.

**G.** The grown colonies are always small. The medium stimulates rapid but not luxurious growth. This asks for rather considerable magnification in order to see the colonies distinctly. Magnifying 50 times will do for practically every purpose. The simple magnifier originally used and sufficient for observing colonies of common bacteria, thus has been changed for a simple microscope on the table of which the screw-axis with the flask-holder are mounted (fig. 4). The essential advantage of the method consisting in the spiral movement of the flask along a counting slit (JULIUS, 1938) is maintained by bringing a thin glass disk into the eyepiece on which a clear band is left free between two opaque segments. A crossline



divides the clear field into two parts (fig.5). The parts are so adjusted that by a given length of the tubulus and by the chosen objective and eyepiece the width of the slit corresponds to the effective speed of the screw (1.25 mm in our case). By turning the screw-axis the total surface of the flasks is covered never touching the same place more than once. Practically the whole flask can be examined

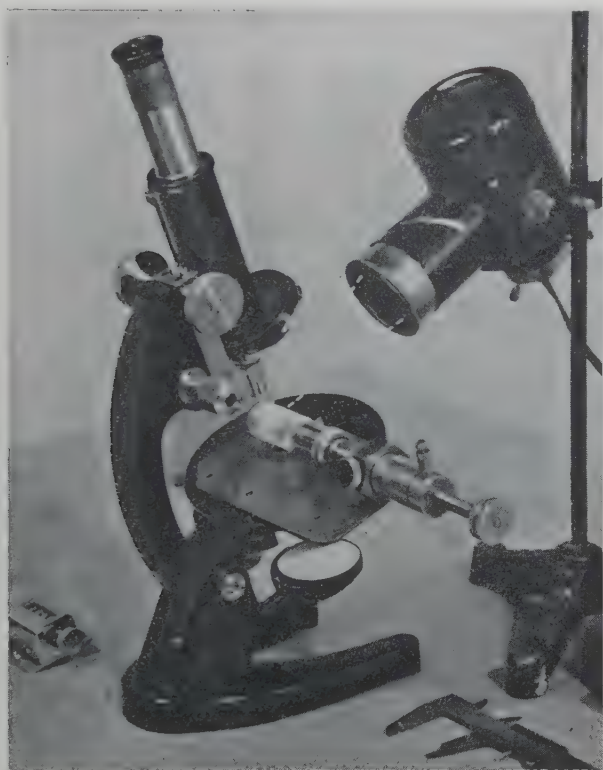


Fig. 4.

without recounting or missing a colony. In order to deal in the agar layer of a few millimeters high with the colonies at different distances from the objective a lens of long focal distance bearing an irisdiaphragm is used, the diaphragm being as much narrowed down as possible. This however asks for a very intense illumination. A beam of light from aside by a lens focussed on the flask just under the objective, answered the purpose and showed bright luminous colonies on a light-grey background. To eliminate troublesome scattering



effects of small scratches in the glass surface of the flask it was thinly coated with liquid paraffin. It may be advantageous to add a trace of neotetrazoliumchloride to the medium (100  $\gamma$ /ml) in order to make the colonies stain a faint red which helps to avoid mistaking dead particles for colonies. If at the same time by the mirror of the microscope a beam of green light is thrown on the flasks an agreeable contrast of red colonies on a green background will result. After some routine however it will suffice to work with unscreened light and without neotetrazoliumchloride.

Like in the original technique the number of colonies in one flask which still can be accepted for accurate working amounts to about 10.000. This gives for ample allowance in case the number of viable units per drop has been grossly mis-estimated. Dilutions can be suitably made in a solution of 0.1% phospholipides in 0.9% NaCl. It better protects viability than does pure salt solution. If the number of colonies is high, it appeared to be sufficient to count the colonies over a limited number of rotations so that about 200 colonies

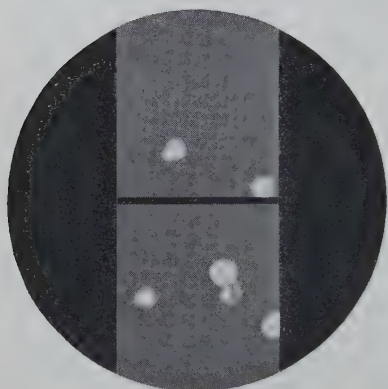


Fig. 5.

are counted and to find the total number by the following formula:

$$n = d \times \frac{a}{o} \times \frac{l}{s} \times L \text{ in which means}$$

$n$  = number of viable units per drop of bacterial suspension under investigation.

$d$  = dilution factor.

$a$  = number of colonies counted.

$o$  = number of rotations.

$s$  = speed of screw (1.25 mm).

$L$  = length of the agar cylinder in mm.

In case of lower numbers of colonies the uneven glass surface near bottom and neck of the flasks may cause some irregularities, so never more than the colonies in 20 rotations are counted and the values substituted in the formula. The length of the agar cylinder is measured by a pair of sliding compasses. Both number of colonies

and number of rotations are registered separately on one of the well-known small mechanical counting devices.

Cleaning the flasks is done by unstopping, boiling in a large sauce-pan in ample water with a suitable detergent and final rinsing. From time to time chemical cleaning is done in strong chromosulfuric solution, followed by very thorough rinsing.

To demonstrate the reliability of the method the following experiment may be an example.

From a ten days old culture of the homogeneously growing D 328 strain the following dilutions were made by standard drop technique: 1/400; 1/1000; 1/2000; 1/4000; 1/10.000; 1/20.000; 1/40.000 and 1/100.000. The roller flasks received as usual one standard drop of dilutions. After 7 days incubation counts were done and by means of the above mentioned formula the original number of viable units per drop was calculated. The results were:

| Dilutions | Average number of colonies per flask | calculated number of viable units per drop of the original culture | logarithm of count (for the sake of logarithmic "curves") |
|-----------|--------------------------------------|--------------------------------------------------------------------|-----------------------------------------------------------|
| 1/400     | 6701                                 | $268 \times 10^4$                                                  | 6.43                                                      |
| 1/1000    | 2530                                 | $253 \times 10^4$                                                  | 6.40                                                      |
| 1/2000    | 909                                  | $242 \times 10^4$                                                  | 6.38                                                      |
| 1/4000    | 419                                  | $223 \times 10^4$                                                  | 6.35                                                      |
| 1/10.000  | 141                                  | $188 \times 10^4$                                                  | 6.27                                                      |
| 1/40.000  | 38                                   | $200 \times 10^4$                                                  | 6.30                                                      |
| 1/100.000 | 16                                   | $209 \times 10^4$                                                  | 6.32                                                      |

The experiment illustrates the good reliability of the method. By the way it may be noted that even with so high a number of colonies per flask (more than  $\pm 6000$ ) as in the specimen experiment, no crowding effect will interfere.

### S u m m a r y.

The publication of CHRISTENSEN, ROBINSON and WIDDICOMBE (1953) was an occasion to draw renewed attention to the method of counting viable germs in rolling flasks since 1938 used in the Laboratory of Hygiene, State University at Utrecht and elsewhere (JULIUS, 1938). The way in which technique and medium are adapted to the viable counting of tubercle bacteria is described.

The author wishes to express his gratitude to Mr. A. KERSSEN whose technical skill was of great value in constructing the apparatus and who specially suggested to give slight inclination to the axes of the setting rotators.

### References.

- CHRISTENSEN, A. P., ROBINSON, M. and WIDDICOMBE, M. 1953. Bull. World Health Org. **9**, 29.
- CRANDALL, W. D. 1949. Am. Rev. Tub. **59**, 325.
- FENNER, F. 1951. Am. Rev. Tub. **64**, 353.
- JENNINGS, F. B. 1926. J. Inf. Dis. **39**, 310.
- JULIUS, H. W. 1938. Antonie van Leeuwenhoek **5**, 28.
- PANNISER, M. and MARCIL, B. 1953. Ann. Inst. Past. **84**, 841.
- SMITH, D. G. 1947. Proc. Soc. exp. Biol. Med. **64**, 36.
- VANDIVIERE, J., MAC. GENTRY, W. M. and STUART-WILLIS, H. 1952. Am. Rev. Tub. **66**, 95.
- WOLINSKY, E. and STEENKEN, W. 1947. Am. Rev. Tub. **55**, 281.
- YEGIAN, D. and BUD, V. 1951. Am. Rev. Tub. **64**, 81.
- YOUMANS, G. P. 1946. J. Bact. **51**, 703.
-

(Hôpital de l'Université à Leyde, Clinique des Maladies Internes; Laboratoire de Recherches de la N.V. Koninklijke Pharmaceutische Fabrieken v/h Brocades-Stheeman & Pharmacia, Amsterdam).

## INFLUENCE DE L'ACTION COMBINÉE DU P.A.S. ET DE L'HYDRAZIDE ISONICOTINIQUE (INH) SUR L'AUGMENTATION DE LA RÉSISTANCE DE BACILLES TUBERCULEUX IN VITRO <sup>1)</sup>

par

**A. J. ZWART VOORSPUIJ**

(Reçu le 8 Février 1954).

C'est un fait notoire que l'administration d'une association d'agents antibactériels peut amener un effet tardif sur l'augmentation de la résistance de micro-organismes, vis-à-vis de ces agents. La littérature clinique, par rapport à la tuberculose, appelle surtout l'attention sur l'association de la streptomycine et du P.A.S. Les expérimentations décrites ci-dessous avaient pour but de vérifier s'il était possible de démontrer in vitro une retardation dans l'apparition de la résistance, à l'emploi d'une association P.A.S. et INH.

### PARTIE EXPERIMENTALE.

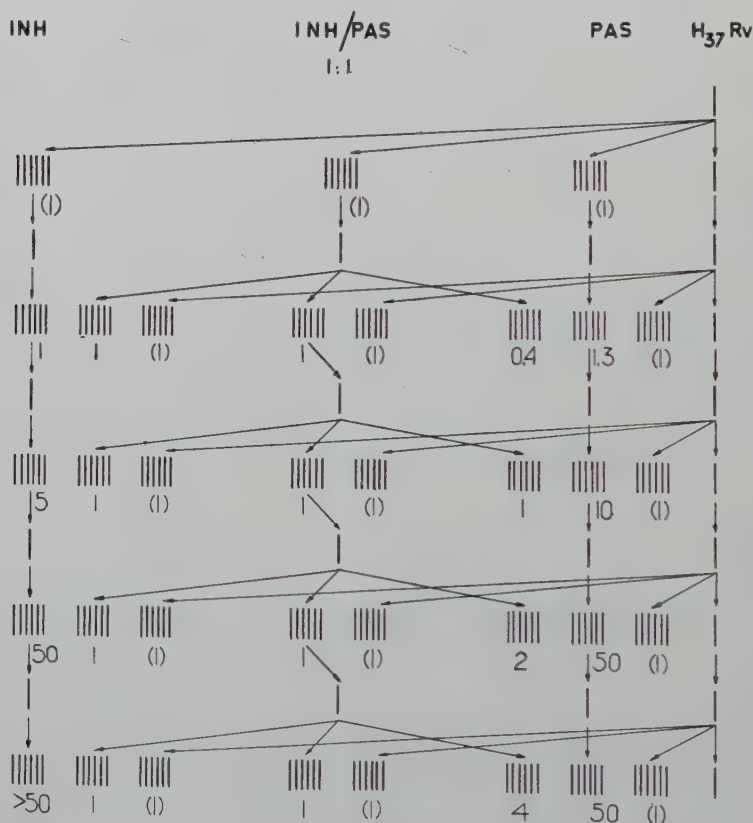
Dans les expérimentations on a fait usage de la souche H37 RV et d'un milieu modifié de Beeuwkes (ZWART VOORSPUIJ et NASS, 1953).

Le tableau ci-joint indique la composition exploratrice. La souche de départ futensemencée sur des séries contenant des concentrations ascendantes de P.A.S., d'INH et d'un mélange consistant en des quantités de poids égales de P.A.S. et d'INH. La souche de départ fut aussi portée dans un milieu sans aucune adjonction. Après 12 jours de croissance, on a déterminé la valeur limite — la concentration la plus faible produisant encore une inhibition complète de la croissance — et les bacilles poussés sur le médium, ayant la concentration précédant à la valeur limite, furentensemencés sur milieu de Loewen-

---

<sup>1)</sup> Les travaux de recherches furent supportés en partie par Riker Laboratories Inc., Los Angeles, California.

stein. Les cultures ainsi créées ont servi de point de départ pour de nouveaux ensemencements de la façon indiquée dans le tableau. Les bacilles s'étant formés sur la concentration la plus élevée furent donc ensemencés régulièrement, tout comme la souche de départ qui fut également examinée à côté de chaque série. Tous les ensemencements indiqués dans le tableau sur le même niveau horizontal furent exécutés le même jour sur des milieux consistant en le même matériel de départ. L'ensemencement lui-même se faisait avec le même volume d'une suspension bactérienne modérément troublante et — là où c'était possible — avec la même suspension.



#### RESULTATS ET CONSIDERATIONS.

Les résultats obtenus ont été indiqués dans le tableau comme le quotient de la valeur limite trouvée et les résultats obtenus avec la



souche originale sur la même série; par conséquent, cette dernière est par définition égale à un (indiqué comme (1)).

Nous constatons alors une prompte augmentation de la résistance à des ensemencements successifs sur des cultures additionnées d'INH et de P.A.S., aucune augmentation de la résistance, par rapport au mélange, ne se manifeste. Les bacilles qui subissent l'influence du mélange, ne montrent pas non plus une augmentation de la résistance à l'égard d'INH alors que celle, à l'égard de P.A.S., ne se déclare que beaucoup plus lentement. Il s'explique qu'il ne faut pas perdre de vue que les concentrations des composants isolés auxquels sont exposés les bacilles dans le mélange ne sont pas égales (plus petites!) à celles que subissent les bacilles qui ont été transplantés de milieux de cultures qui contiennent exclusivement soit du P.A.S., soit de l'INH. Tout cela cependant ne fait que confirmer le fait qu'il y a du moins une combinaison (50/50) d'INH et de P.A.S. qui a la faculté d'empêcher complètement la croissance de bacilles tuberculeux sans que l'augmentation de la résistance ne se manifeste au même degré que c'est le cas en faisant usage des éléments isolés. Les ensemencements dans les autres milieux comme indiqués dans le tableau ont été continués entretemps jusqu'au nombre total de huit fois. Les résultats étaient sensiblement les mêmes. S'il s'agit de mélanges de substances, il n'y a pas de simple connexion entre les résultats des expérimentations *in vitro* et ceux obtenus dans la thérapeutique clinique. Au contraire, cette connexion est très compliquée; toutefois, les résultats de nos expérimentations justifient d'y voir une indication que, dans la clinique aussi, l'administration de l'association INH et P.A.S. — vis-à-vis de l'augmentation de la résistance — pourrait présenter des avantages réels, supérieurs à ceux que l'on obtient en administrant les éléments isolés.

### R é s u m é.

Un examen comparatif *in vitro* fait ressortir que l'augmentation de la résistance des bacilles tuberculeux vis-à-vis d'un mélange d'INH et de P.A.S. ne se présente pas ou seulement lentement après des ensemencements successifs sur des milieux qui contiennent ce mélange. Ceci à l'opposé de bacilles exposés à l'influence de ces deux éléments isolés. Les bacilles qui subissent l'influence du mélange font voir également une légère augmentation de la résistance à l'égard des composants isolés.

L'auteur exprime ses remerciements à M.M. L. H. Bokma et F. A. Heemskerk pour l'aide d'ordre technique dont il a pu profiter.

### Bibliographie.

ZWART VOORSPUIJ, A. J. et NASS, C. A. G. 1953. *Antonie van Leeuwenhoek* **19**, 225.

---

(From the National Institute of Public Health, Utrecht, and the Regional Laboratory of Public Health, Enschede, The Netherlands).

## A NEWLY DISCOVERED *SALMONELLA* TYPE (*S. ISRAEL*)

by

A. CLARENBURG, A. MARSEILLE and H. H. VINK

(Received February 13, 1954).

In April 1953 a culture isolated from the stools of an 80-year-old woman of Glanerbrug near Enschede was examined at the National Salmonella Centre in our Institute. The patient had suffered at the age of 12 from typhoid and for the last 26 years had been suffering from colitis ulcerosa, with intermittent complaints.

The culture possessed the morphological and biochemical characteristics typical of the genus *Salmonella*. The organisms were motile and Gram-negative. The biochemical behaviour was as follows: No fermentation of inositol, lactose, salicin and sucrose. No production of indole, no liquefaction of gelatin and no hydrolysis of urea. Rapid fermentation of arabinose, dulcitol, glucose (with gas), maltose, mannitol, rhamnose, sorbitol, trehalose, and xylose. Production of  $H_2S$ . Nitrates were reduced, the Voges-Proskauer reaction was negative, and the methyl-red reaction positive.

Examined serologically the organism was agglutinated to titre by *S. dublin* O serum and in absorption tests removed all agglutinins from that serum. Therefore the culture belonged to group D of the genus *Salmonella*; it contained the somatic antigens 9,12<sup>1</sup>). The H antigens were diphasic. Phase 1 was agglutinated to the titre of, and in absorption tests removed all agglutinins from, serum derived from phase 1 of *S. eastborne* (e,h). Phase 2 was agglutinated to the titre of, and in absorption tests removed all agglutinins from, serum derived from phase 2 of *S. san diego* (e,n,z<sub>15</sub>).

---

<sup>1</sup>) In accordance with a recent decision of the Enterobacteriaceae Subcommittee of the Nomenclature Committee of the International Association of Medical Bacteriologists the O antigens of *Salmonella* types are expressed by Arabic numerals.

The antigenic formula of the type examined was therefore 9, 12: e,h-e,n,z<sub>15</sub>.

Since a similar type had not been described so far, the culture was sent to F. KAUFFMANN, Director of the International Salmonella Centre (Copenhagen), who confirmed our diagnosis. He informed us, however, that a short time ago the same type had already been examined by P. R. EDWARDS (U.S.A.). The latter informed us that he had received an identical *Salmonella* strain, isolated from the stools of a normal human carrier in Israel. He had found the same antigenic formula and called the new type: *Salmonella israel*.

#### S u m m a r y.

A description is given of a *Salmonella* type isolated from the faeces of an adult female suffering from colitis ulcerosa. The antigenic formula was 9, 12: e,h-e,n,z<sub>15</sub>. A short time before this examination an identical culture, isolated from a normal human carrier in Israel, was examined by Dr P. R. EDWARDS (U.S.A.). He called the new type *Salmonella israel*.

---

(Institut Agronomique, Université de Louvain, Belgique).

## CHROMATOGRAPHIE DES COMPOSANTS TERNAIRES DU MYCELIUM DE *PENICILLIUM BREVI-COMPACTUM*

par

**PAUL GODIN** <sup>1)</sup>

(Reçu le 4 Mars 1954).

Nous avons donné ailleurs (GODIN, 1953) les différentes substances ternaires trouvées par chromatographie du milieu de culture de *P. brevi-compactum*. Dans cette note, nous exposons les résultats de l'analyse chromatographique du mycélium lui-même. Certains composés produits par la moisissure, et tout particulièrement certains intermédiaires du métabolisme ou dérivés de ceux-ci, sont en effet susceptibles de n'être trouvés que dans le mycélium, soit qu'ils ne diffusent point de la cellule dans le milieu extérieur, ou qu'ils soient par trop dilués dans la solution de métabolisme pour y être décelés. Il est donc possible que la chromatographie du mycélium nous apporte des renseignements nouveaux sur le chimisme de la moisissure.

### MÉTHODES EXPÉRIMENTALES.

La souche de *P. brevi-compactum* étudiée provient de la mycothèque de BIOURGE, Laboratoire de Microbiologie, Université de Louvain, où elle est cataloguée sous le numéro Db 182A.

La moisissure est ensemencée dans des erlenmeyers de 500 ml de capacité contenant 200 ml de milieu Czapek-Dox ou Raulin-Thom, dans le cas des cultures en surface, et 50 ml de milieu Czapek-Dox, dans le cas des cultures agitées. L'incubation a lieu à 25° C.

Le mycélium est prélevé pour l'analyse en pleine période de développement, alors que  $\frac{1}{4}$  environ de la quantité initiale du glucose du milieu de culture reste encore à consommer.

---

<sup>1)</sup> Chargé de Recherches du Fond National de la Recherche Scientifique; Adresse actuelle: Laboratoire de la Sté Steinbach, Malmédy, Belgique.



## PRÉPARATION DES SOLUTIONS A CHROMATOGRAPHIER.

Obtention des composants du mycélium solubles dans l'éther.

Le mycélium provenant de 10 cultures est rincé à l'eau distillée, puis immédiatement extrait durant 30 minutes par 600 ml d'eau distillée bouillante. Après refroidissement, on filtre la solution, l'acidifie à pH inférieur à 3 par  $\text{H}_2\text{SO}_4$  dilué et l'extrait durant une nuit par l'éther sulfurique, dans un extracteur continu. L'extrait éthéré est concentré à petit volume et chromatographié. On y recherche les acides organiques, les composés cétoniques et les substances phénoliques.

Obtention des sucres, dérivés des sucres et autres substances hydrosolubles.

Le mycélium provenant de 10 cultures est rincé à l'eau distillée, puis directement extrait durant 30 minutes par 600 ml d'eau distillée bouillante. Après refroidissement, on filtre la solution et la fait passer par une colonne de Zeo-karb ( $\text{H}^+$ ), résine cationique qui retient les cations, les acides aminés et autres corps amphothères. La solution ainsi traitée traverse ensuite une colonne d'Amberlite IR-4B( $\text{CO}_3^-$ ) qui capte les anions. La solution obtenue ne renferme donc que des corps neutres et constitue, après concentration sous vide, la fraction I. Les anions retenus par la résine anionique sont élués par l'acide formique à 5%. Cette élution est réalisée en déversant la résine de la colonne dans un vase de Berlin contenant un excès de la solution d'acide formique. Après agitation, lorsque cesse le dégagement de  $\text{CO}_2$ , on remet la résine dans la colonne et opère comme normalement. L'éluat et les eaux de rinçage, après concentration sous vide, constituent la fraction II. Il est indispensable de faire agir la solution d'acide formique sur l'Amberlite IR-4B( $\text{CO}_3^-$ ) en dehors de la colonne, sinon le  $\text{CO}_2$  qui se dégage, arrête la percolation de liquide ou rend l'élution fort imparfaite. On a choisi d'opérer avec la forme  $\text{CO}_3^-$  d'une résine anionique faible pour éviter toute dégradation des sucres, phénomène signalé par PHILLIPS et POLLARD (1953).

Dans la fraction I, on a recherché les sucres, polyols et composés cétoniques neutres; dans la fraction II, les acides dérivés des sucres, leurs lactones et les acides cétoniques.

## Méthodes chromatographiques.

Le papier employé est le Whatman no 4. On chromatographie toujours par la méthode ascendante, sauf si on utilise le phénol comme solvant; dans ce cas, on chromatographie par voie descendante.

Les systèmes de solvants:

pour les sucres et leurs dérivés (acides, polyols, lactones), on emploie le phénol saturé d'eau non additionné d'ammoniaque (symbole Ph) ou la couche surnageante d'un mélange de 4 volumes de n-butanol, de 1 volume d'acide acétique et de 5 volumes d'eau (symbole BAc), solvants proposés par PARTRIDGE (1948).

pour les acides non volatils et les substances cétoniques, on utilise le mélange de CHOW (1951): 2 volumes de n-butanol saturé d'eau et 1 volume d'acide formique à 90% (symbole BF), ou encore le solvant BAc décrit plus haut.

pour chromatographier les acides phénoliques, on se sert du système de solvants préparé en mélangeant 5 volumes de chloroforme à 3 volumes d'acide acétique et en ajoutant goutte à goutte à ce mélange la quantité d'eau tout juste suffisante pour atteindre la saturation sans la dépasser (symbole Chl).

Les révélateurs:

les sucres et leurs dérivés sont mis en évidence soit par l'oxalate acide d'aniline de HORROCKS et MANNING (1949), soit par le réactif au naphtorésorcinol de PARTRIDGE (1948), soit encore par le dichlorhydrate d'o-phénylènediamine proposé par LANNING et COHEN (1951).

les lactones, notamment celles des acides oniques, sont révélées par la méthode d'ABDEL-AKHER et SMITH (1951).

les polyols neutres (sucres et alcools polyvalents) qui sont recherchés dans la seule fraction neutre (fraction I), sont mis en évidence par le procédé de HOCKENHULL (1953) et par celui de GODIN (1954).

les acides sont décelés par le vert de bromocrésol à 0,05% dans l'éthanol.

les substances cétoniques sont révélées par le réactif de Brady (2,4-dinitrophénylhydrazine).

les substances phénoliques sont mises en évidence par le chlorure ferrique à 0,5%, par le réactif de Pauly, suivant la méthode de BOLLING *et al.* (1949) ou, de préférence, par le réactif à la p-nitraniline proposé par BRAY *et al.* (1950) et amélioré par MAYAUDON (1954).

Les composés qui n'ont pas été trouvés dans le commerce ont été synthétisés par nous. Tel est le cas des acides l-arabonique, d-xylo-

nique et d-ribonique préparés à partir des pentoses correspondants par la méthode de HOENIG et RUZICKA (1930) et des acides l-érythronique et d-thréonique obtenus respectivement aux dépens de l-arabinose et de d-xylose par la méthode de SCHEUING et KONZ (1949). Quant aux l-arabitol, d-xylitol et d-adonitol, ils ont été obtenus par hydrogénation catalytique sous pression des pentoses correspondants.

## LES RÉSULTATS EXPÉRIMENTAUX.

### Composants du mycélium solubles dans l'éther.

**Cultures en surface.** L'extrait éthéré du mycélium s'étant développé sur le milieu Czapek-Dox renferme les substances identifiées que voici:

— **l'acide citrique** faiblement concentré. Caractérisation: acide.  $R_f$  : 0,33 dans BF.

— **l'acide malique** dont la concentration est sensiblement égale à celle du premier cité. Caractérisation: acide.  $R_f$  : 0,41 dans BF.

— **l'acide succinique** plus dilué que les précédents. Caractérisation: acide.  $R_f$  : 0,67 dans BF.

— **l'acide fumarique** à même concentration que le précédent. Caractérisation: acide.  $R_f$  : 0,82 dans BF.

— **l'acide 3,5-dihydroxyphthalique** à faible concentration. Caractérisation: acide; tache pourpre-amarante au  $\text{FeCl}_3$ ; tache jaune intense avec le réactif de Pauly et celui à la p-nitraniline.  $R_f$  dans Chl : 0,1.

— **l'acide phénolique**  $\text{C}_{10}\text{H}_{10}\text{O}_6$  très faiblement concentré. Caractérisation: acide; cétonique; tache pourpre-amarante au  $\text{FeCl}_3$ ; tache jaune pâle avec le réactif de Pauly et jaune brunâtre avec celui à la p-nitraniline.  $R_f$  dans Chl : 0,33.

— **l'acide phénolique**  $\text{C}_{10}\text{H}_{10}\text{O}_7$  très faiblement concentré. Caractérisation: acide; cétonique; tache pourpre-amarante au  $\text{FeCl}_3$ ; tache rosée, virant immédiatement au beige et ultérieurement au brun avec le réactif de Pauly; tache beige pâle virant ensuite au brun avec le réactif à la p-nitraniline.  $R_f$  dans Chl : 0,46.

— **l'acide phénolique**  $\text{C}_{10}\text{H}_{10}\text{O}_5$  faiblement concentré. Caractérisation: très faiblement acide; cétonique; tache pourpre pâle avec le  $\text{FeCl}_3$ ; tache jaune avec le réactif de Pauly et celui à la p-nitraniline.  $R_f$  dans Chl : 0,63.

— **l'acide mycophénolique** à l'état de traces. Ce corps ne réagit pas avec le réactif de Pauly, ni avec celui à la p-nitraniline. Caractérisation: tache violet-bleu au  $\text{FeCl}_3$ .  $R_f$  dans Chl : 0,97.

Si la moisissure s'est développée sur le milieu Raulin-Thom, l'extrait éthéré renferme les seuls composés identifiés que voici:

— **l'acide phénolique**  $\text{C}_{10}\text{H}_{10}\text{O}_6$  en quantité moyenne.

— **l'acide phénolique**  $\text{C}_{10}\text{H}_{10}\text{O}_7$  en grande quantité.

— **l'acide phénolique**  $\text{C}_{10}\text{H}_{10}\text{O}_5$  en faible quantité.

— **l'acide 3,5-dihydroxyphthalique** en grande quantité.

— **l'acide mycophénolique** en quantité assez minime.

**Cultures agitées.** Dans le cas des cultures agitées, l'extrait éthéré contient les substances identifiées suivantes:

- l'acide citrique très dilué.
- l'acide malique à même concentration que le précédent.
- l'acide succinique en quantité notable.
- l'acide fumarique légèrement moins concentré que le précédent.
- l'acide 3,5-dihydroxyphthalique à faible concentration.
- les acides phénoliques  $C_{10}H_{10}O_6$  et  $C_{10}H_{10}O_7$  très faiblement concentrés.
- l'acide mycophénolique faiblement concentré.
- des traces d'acide phénolique  $C_{10}H_{10}O_5$ .

Signalons enfin que l'extrait éthéré du mycélium, tant des cultures en surface que des cultures agitées, renferme de faibles quantités de plusieurs substances phénoliques non identifiées, autres donc que celles signalées par CLUTTERBUCK *et al.* (1932).

## Substances neutres trouvées dans la fraction I.

**Cultures en surface.** Le mycélium qui se développe sur le milieu Czapek-Dox renferme les composés identifiés que voici:

- le glucose en abondance. Caractérisation: tache brune avec l'oxalate acide d'aniline.  $R_f$  : 0,39—0,41 dans Ph.
- l'arabinose faiblement concentré. Caractérisation: tache rose avec l'oxalate acide d'aniline.  $R_f$  : 0,58—0,60 dans Ph.
- le ribose faiblement concentré. Caractérisation: tache rose avec l'oxalate acide d'aniline.  $R_f$  : 0,66—0,68 dans Ph.
- le fructose faiblement concentré. Caractérisation: tache rose avec le réactif au naphtorésorcinol; tache gris-vert très foncée avec le réactif à la vanilline.  $R_f$  : 0,57—0,59 dans Ph et 0,16 dans BAc.
- le mannitol en abondance. Caractérisation: polyol; tache mauve-rose avec le réactif à la vanilline.  $R_f$  : 0,49 dans Ph et 0,15 dans BAc.
- le i-érythritol moyennement concentré. Caractérisation: polyol; tache violet pâle avec le réactif à la vanilline.  $R_f$  : 0,67 dans Ph et 0,29 dans BAc.
- le glycérol très faiblement concentré. Caractérisation: polyol; tache bleu-violet avec le réactif à la vanilline.  $R_f$  : 0,75 dans Ph et 0,42 dans BAc.

Lorsque le mycélium se développe sur le milieu Raulin-Thom, on y trouve, dans des proportions sensiblement identiques, les mêmes substances, sauf le fructose qui n'est pas décelé. On met en outre en évidence un pentitol faiblement concentré. Il s'agit soit de l'adonitol, soit de l'arabitol. Caractérisation: polyol; tache bleu-mauve avec le réactif à la vanilline.  $R_f$  : 0,59—0,61 dans Ph et 0,21 dans BAc. Ce sont également les valeurs trouvées pour l'arabitol et l'adonitol dont les  $R_f$  sont pratiquement identiques dans les solvants employés. Les  $R_f$  du xylitol sont 0,62—0,65 dans Ph et 0,185 dans BAc, ce qui permet de le différencier de la substance trouvée. Il est possible que le pentitol se trouve également dans le mycélium des cultures en surface sur le milieu Czapek-Dox. Le fructose qui y est présent empêche toutefois de le déceler, car, d'une part, son  $R_f$  est peu différent de celui du pentitol et, d'autre part, il donne avec le réactif de Hockenhull la réaction des polyols et avec le réactif à la vanilline une tache gris-vert très foncée qui empêche de déceler la tache plus pâle du pentitol.



**Cultures agitées.** Le mycélium des cultures agitées renferme, dans des proportions sensiblement identiques, tous les corps neutres décelés dans le mycélium des cultures en surface sur le même milieu Czapek-Dox, sauf le fructose qui n'est pas mis en évidence. S'ajoute à ces substances le pentitol déjà trouvé dans le mycélium des cultures en surface sur le milieu Raulin-Thom.

### Substances acides mises en évidence dans la fraction II.

**Cultures en surface.** On décèle les composés identifiés suivants, présents dans le mycélium qui s'est développé sur le milieu Czapek-Dox: — l'**acide gluconique** en abondance. Caractérisation: acide; tache jaune fugace au  $\text{FeCl}_3$ .  $R_f$  dans BF : 0,15.

— l'**acide 2-cétogluconique** très faiblement concentré. Caractérisation: tache rose à l'oxalate acide d'aniline; tache brun-doré au dichlorhydrate d'ophténylènediamine.  $R_f$  dans Ph : 0,19.

— l'**acide glucuronique** très faiblement concentré. Caractérisation: tache jaune-brun à l'oxalate acide d'aniline, virant ultérieurement au rose.  $R_f$  dans Ph : 0,1—0,2.

— la **gluconolactone** fort concentrée. Caractérisation: lactone.  $R_f$  : 0,56 dans Ph et 0,37 dans BAc.

— mentionnons aussi une **lactone non identifiée** de  $R_f$  0,65 dans BAc et 0,73 dans Ph, qui n'est ni une hexono-, ni une pentono-, ni une tétrono-lactone.

Lorsque le mycélium provient d'une culture sur le milieu Raulin-Thom, on trouve les mêmes substances, sauf l'acide 2-cétogluconique qui n'est pas mis en évidence.

**Cultures agitées.** Le mycélium des cultures agitées renferme les mêmes substances que celles trouvées dans le mycélium des cultures en surface sur le même milieu Czapek-Dox, sauf l'acide 2-cétogluconique non décelé.

### DISCUSSION.

Les substances trouvées dans le mycélium, mais qui n'ont point été signalées dans les notes traitant de l'analyse du milieu de culture (GODIN, 1953), sont: le fructose, le mannitol, l'arabitol ou l'adonitol, l'i-érythritol et le glycérol. Toutefois, en appliquant au milieu de culture les mêmes techniques que celles employées pour la recherche des sucres et dérivés des sucres du mycélium, on a pu y déceler des quantités abondantes de mannitol et de très faibles quantités de i-érythritol. Par contre, le fructose, le pentitol et le glycérol n'ont pu y être mis en évidence.

Le glycérol, quoique produit par plusieurs moisissures, n'avait jamais été obtenu, du moins à notre connaissance, à partir d'un *Penicillium*. L'i-érythritol, par contre, a déjà été trouvé par OXFORD



et RAISTRICK (1935) dans le mycélium de *P. brevi-compactum* et le mannitol est connu pour être un produit du métabolisme de nombreuses moisissures. Quant au pentitol, c'est la première fois qu'on signale l'existence d'un tel corps chez une moisissure.

La mise en évidence de ces substances nouvelles n'infirme pas les hypothèses avancées dans une note précédente (GODIN, 1953) pour expliquer la production des autres composés par la moisissure. On y suggérerait l'existence chez *P. brevi-compactum* de l'oxydation directe du glucose et d'un processus de transformation des acides organiques analogue au cycle de KREBS. La présence dans le mycélium des acides gluconique, 2-cétogluconique et glucuronique, du ribose, de l'arabinose, d'un pentitol et de l'i-érythritol fait en effet supposer que l'oxydation directe du glucose est réalisée par la moisissure. De même, la coexistence dans le mycélium des acides citrique, succinique, fumarique et malique peut s'expliquer en admettant que se déroule chez la moisissure un processus analogue au cycle de KREBS. A la suite de la découverte du fructose et du glycérol parmi les produits du métabolisme du glucose, il semble qu'on puisse ajouter une nouvelle hypothèse: celle qui suppose l'existence de la glycolyse chez *P. brevi-compactum*.

### R e m e r c i e m e n t s .

L'auteur remercie le Prof. P. SIMONART de l'Université de Louvain, de tout l'intérêt qu'il a porté à ces recherches et de la généreuse hospitalité qu'il lui a accordée dans son laboratoire. Il remercie également le Prof. J. C. JUNGERS de la même Université et Monsieur H. R. DEBUS, L.S.Ch., qui lui ont aimablement préparé les pentitols.

### R é s u m é .

L'analyse chromatographique du mycélium et du milieu de culture de *P. brevi-compactum* a permis d'identifier plusieurs substances ternaires nouvelles, produites par la moisissure: le fructose, le mannitol, l'arabitol ou l'adonitol, et le glycérol. Les résultats obtenus font l'objet d'une discussion.

### S u m m a r y .

Chromatographic analysis of *P. brevi-compactum* mycelium and culture medium has revealed different new ternary substances produced by that mould: fructose, mannitol, arabitol or adonitol and glycerol.

## Bibliographie.

- ABDEL-AKHER, M. et SMITH, F. 1951. J. Am. Chem. Soc. **73**, 5859.  
BOLLING, D., BLOCK, R. J. et SOBER, H. A. 1949. Federation Proc. **8**, 185.  
BRAY, H. G., THORPE, W. V. et WHITE, K. 1950. Biochem. J. **46**, 271.  
CHOW, K. Y. 1951. Mémoires Coll. Inst. Agronomique Univ. Louvain **4**.  
CLUTTERBUCK, P. W., OXFORD, A. E., RAISTRICK, H. et SMITH, G. 1932.  
Biochem. J. **26**, 1441.  
GODIN, P. 1953. Biochim. Biophys. Acta **11**, 114, 119.  
GODIN, P. 1954. Nature (sous presse).  
HOCKENHULL, D. J. D. 1953. Nature **171**, 982.  
HOENIG, M. et RUZICKA, W. 1930. Ber. **63**, 1648.  
HORROCKS, R. H. et MANNING, G. B. 1949. Lancet **256**, 1042.  
LANNING, M.C. et COHEN, S. T. 1951. J. Biol. Chem. **189**, 109.  
MAYAUDON, J. 1954. Mémoires Coll. Inst. Agronomique Univ. Louvain **9**.  
OXFORD, A. E. et RAISTRICK, H. 1935. Biochem. J. **29**, 1599.  
PARTRIDGE, S. M. 1948. Biochem. J. **42**, 238.  
PHILLIPS, J. D. et POLLARD, A. 1953. Nature **171**, 119.  
SCHEUING, G. et KONZ, W. 1949. Ber. **82**, 92.
-

(Laboratoire de Bactériologie de l'Université Libre de Bruxelles, Belgique).

## LE PHENOMENE DU DOUBLE ANNEAU DANS L'ETUDE DES PHAGOSTATIQUES <sup>1)</sup>

par

**J. H. MOLRON**

(Reçu le 13 Février 1954).

NICOLLE et MIMICA (7) déposent au centre d'une boîte de Pétri, ensemencée avec un mélange de germes et du bactériophage correspondant, une goutte de l'antiseptique dont ils veulent étudier l'action sur la bactériophagie. Après un séjour de la plaque à 37° C., pendant 24 heures, ils observent trois zones: 1) une zone centrale entièrement vierge; 2) un anneau de culture microbienne sans tache de bactériophage; 3) une zone périphérique où le phage a exercé son action habituelle sur les germes sensibles et où ne poussent que quelques colonies résistantes.

MUTSAARS (6), en utilisant une variante de cette technique, a pu mettre en évidence que la cystéine détermine plusieurs zones sur une plaque de gélose synthétique. Il a observé, en allant du centre à la périphérie: 1) un effet bactériostatique; 2) un effet antibactériophage; 3) un effet se traduisant par la disparition de toute colonie de colibacille, des colonies résistantes au bactériophage ne se retrouvant qu'en dehors d'une zone où la lyse a été complète.

SPIZIZEN, HAMPIL et KENNEY (8) ont confirmé, dans un travail récent, l'action inhibitrice de la cystéine sur certains bactériophages anticoli. Nous nous sommes attaché à rechercher 1) si la cystéine était le seul agent capable de déterminer l'apparition du double anneau ou si, au contraire, ce phénomène pouvait être provoqué par d'autres substances bactéricides ou bactériostatiques; 2) laquelle des deux hypothèses proposées était valable pour expliquer l'effet se traduisant par la disparition des colonies de colibacilles

---

<sup>1)</sup> Ce travail a fait l'objet de deux notes préliminaires présentées à la Société belge de Biologie (5).

résistants: action directe de la substance étudiée sur ceux ci ou exaltation de la virulence du bactériophage.

#### TECHNIQUE ET MILIEU.

A partir d'un godet de porcelaine poreuse <sup>1)</sup>, nous laissons diffuser pendant 24 heures, dans une boîte de Pétri contenant du milieu synthétique (M9) gélosé à 2%, la substance dont nous voulons étudier l'action sur la bactériophagie. Nous coulons ensuite sur cette plaque 5 cc du même milieu, mais gélosé à 0,7%, maintenu fluide à 55° C., et auquel nous avons incorporé 1 cc d'un mélange constitué, en parties égales, d'une culture de 24 heures en milieu minéral glucosé d'une souche de colibacille B et d'une suspension de bactériophage faiblement dilué en bouillon. Ces dilutions aux concentrations  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , sont effectuées à partir du filtrat, sur bougie L III, d'une culture de coli B en bouillon, agée de 24 heures et contaminée, dès l'ensemencement, par le phage étudié. Nous avons employé, dans nos expériences préliminaires, tous les phages de la série T de Delbrück qui ont été très complètement étudiés en Amérique quant à leur morphologie, leurs propriétés antigéniques, l'aptitude qu'ont les germes coli B à résister à leur action. Les bactériophages de petite taille et à grandes plages de lyse sur milieu solide se sont montrés moins favorables à l'obtention des phénomènes que nous recherchions que ceux de grande taille et à petites plages. Il est à noter que le bactériophage anticoli  $\varphi N$ , utilisé par MUTSAARS (7), était lui aussi un phage à petites plages. Nous avons effectué nos expériences avec du bactériophage  $T_2$ .

La composition du milieu minéral glucosé M9 est la suivante (4):

|                                                     |             |       |
|-----------------------------------------------------|-------------|-------|
| Solution A:                                         | $KH_2PO_4$  | 3,0 g |
|                                                     | $MgSO_4$    | 0,2 g |
|                                                     | NaCl        | 0,5 g |
|                                                     | $NH_4Cl$    | 1,0 g |
|                                                     | $Na_2HPO_4$ | 6,0 g |
| Dissoudre dans l'ordre dans 900 cc d'eau distillée. |             |       |
| Solution B:                                         | Glucose     | 4,0 g |
| Dissoudre dans 100 cc d'eau distillée.              |             |       |

Les deux solutions sont stérilisées séparément et mélangées

---

<sup>1)</sup> E. & A. Penicylinders distribued by Fisher Scientific Co, Pittsburgh (Pa.); Eimer & Amend, New York (N.Y.).

extemporanément dans la proportion de 9 et 1. Le pH final est de 7.

**Expérience 1.** Action de la cystéine (solution de cystéine à 10 pour 100 dans de l'eau distillée).

Nous reprenons en fait ici, mais avec un colibacille et un bactériophage différents, l'expérience de MUTSAARS (6).

Nous observons, après un séjour de la plaque à 37° C., pendant 24 heures: 1) une zone entièrement vierge; 2) un anneau constitué par une culture de coli sans trace de bactériophage; 3) un second anneau continuant le premier et constitué par une nappe de colibacille dans laquelle des plages de bactériophages apparaissent et deviennent de plus en plus nombreuses au fur et à mesure que l'on s'éloigne du centre devenant finalement confluentes et dentelant le bord périphérique de l'anneau; 4) une zone claire où la lyse du colibacille a été complète (zone de lyse maximale); 5) une zone tout à fait périphérique contenant des colonies de colibacilles résistants au bactériophage.

**Expérience 2.** Action de la trypaflavine (chlorure de trypaflavine Bayer en solution aqueuse concentrée).

L'action de la trypaflavine comme agent anti microbien et anti bactériophage est suffisamment connue pour qu'il ne soit nécessaire d'y revenir ici.

Si nous laissons diffuser cette substance dans de la gélose synthétique M9, que nous ensemençons suivant la technique décrite ci-dessus, nous observons le phénomène du double anneau avec zone de lyse maximale comme dans l'expérience 1. Les zones sont même beaucoup mieux tranchées et le phénomène est remarquablement net (fig. 1).

**Expérience 3.** Des résultats semblables à ceux des expériences 1 et 2 ont été obtenus en étudiant l'action sur la bactériophagie du rivanol, de la

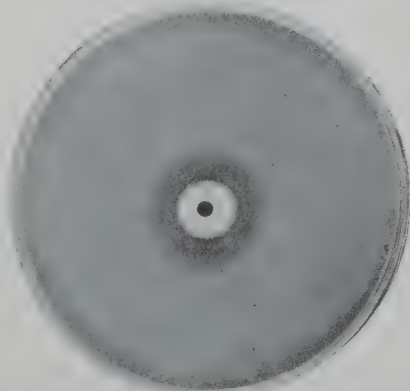


Fig. 1. Plaque de gélose minérale dans laquelle on a laissé diffuser de la trypaflavine et ensemencée avec un mélange de colibacille B et de bactériophage T<sub>2</sub>.

La photo a été prise après un séjour de la plaque pendant 24 heures à 37 C. On distingue, en allant du centre à la périphérie, une zone vierge, un anneau de colibacille sans trace de bactériophage, l'anneau de lyse maximale, une zone où poussent les germes résistants au bactériophage.



mépacrine, du paranitrophénol et de la paranitrophénylglycine en solution concentrée dans de l'eau distillée. Toutefois, la zone de lyse maximale est plus étroite que celle due à l'action de la tryptaflavine.

**Expérience 4.** Influence de la concentration en bactériophage.

Les expériences précédentes ont été effectuées en employant, ainsi qu'il a été dit dans l'exposé de la technique suivie, des dilutions de bactériophage de l'ordre de  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ . Si nous les reprenons en utilisant des dilutions plus élevées de principe lytique ( $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ) nous trouvons:

1) une zone centrale claire, vierge de toute croissance où l'effet bactéricide ou bactériostatique s'est manifesté; 2) une zone où le colibacille pousse sans trace de bactériophage; 3) une zone périphérique où la nappe de germes microbiens est tachetée de plages de bactériophages presque confluentes à la dilution  $10^{-4}$ , mais devenant évidemment de plus en plus rares aux dilutions supérieures. Il n'y a pas de zone de lyse maximale.

**Expérience 5.** Influence de la température.

L'action de la température sur la bactériophagie est bien connue. FLU (3), BERGER et ROESLI (1), BLOCH (2) ont étudié son action sur la multiplication du phage et sur l'apparition de la lyse bactérienne. Nous avons recherché son influence possible sur l'apparition du double anneau.

Nous avons repris les expériences 1, 2 et 3, mais au lieu de placer les plaquesensemencées pendant 24 heures à  $37^{\circ}\text{C}.$ , nous les avons abandonnées pendant 48 heures à  $16^{\circ}\text{C}.$  On note alors: 1) une zone centrale apparemment stérile; 2) une zone où le coli pousse sans trace de phage; 3) une zone périphérique riche en plages de bactériophage mais dont le gazon microbien continue sans interruption la zone 2. L'aspect de la plaque est tout à fait semblable à celui obtenu lors de l'ensemencement de germes et de bactériophage très dilué et, comme dans l'expérience 4, il n'y a pas de zone de lyse maximale.

Si, après un séjour de 48 heures à  $16^{\circ}\text{C}.$ , ces plaques sont portées dans une étuve à  $37^{\circ}\text{C}.$  pendant 24 heures, on observe une ébauche de zone de lyse maximale, tandis que les plages de la zone périphérique semblent s'être multipliées, le gazon microbien devenant discontinu.

Résumons brièvement ces observations. La cystéine, la trypta-

flavine, le rivanol, la mépacrine, le paranitrophénol et la paranitrophénylglycine exercent, en milieu minéral solide, outre une action anticoli B et antibactériophage  $T_2$ , un effet se traduisant par la disparition des colonies de coli résistant au phage  $T_2$ . Certaines conditions de milieu, de température, de richesse en bactériophage sont nécessaires pour que le phénomène du double anneau soit observé. On peut envisager l'apparition de la zone de lyse maximale comme due soit à une action directe de la substance étudiée sur les germes résistants, soit à une exaltation de la virulence du phage.

Pour élucider ce problème, nous avons comparé l'action des divers agents chimiques étudiés plus haut sur les germes coli B normaux et sur les germes coli B devenus résistants au bactériophage  $T_2$ .

Nous avons soumis à l'action d'un excès de particules de bactériophage  $T_2$ , 100 à 1000 millions de colibacilles B. Nous avons ainsi isolé un germe résistant au bactériophage  $T_2$  que nous avons appelé, suivant la terminologie américaine, coli B/2. Le filtrat de cette culture B/2 est inactif sur la souche B: il n'y a donc plus de trace de bactériophage  $T_2$  dans la culture de mutants.

Au centre d'une boîte de Petri contenant de la gélose synthétique encore fluide (milieu M9) nous déposons un godet de porcelaine poreuse puis, de part et d'autre de ce godet et dans le prolongement l'une de l'autre, deux baguettes de verre stériles.

Nous laissons diffuser pendant 24 heures, à partir du godet, une solution concentrée du produit dont nous voulons étudier l'action. Il est bien évident qu'un même gradient règle la vitesse de diffusion à partir du godet dans chacune des deux demi-plaques. Une de celles-ci est alorsensemencée avec 1 cc d'une culture de 24 heures, en milieu M9, d'une souche de colibacille normal, incorporé à 5 cc de milieu minéral glucosé, gélosé à 0,7% et maintenu fluide à 55 °C., l'autre demi-plaque recevant semblablement 1 cc d'une culture de coli B/2. Les lectures sont effectuées après un séjour de 24 heures à 37 °C.

Expérience 6. Nous avons déposé dans le godet central quelques gouttes d'une solution de cystéine à 10% dans de l'eau distillée. A la lecture, on note que l'anneau d'inhibition de la croissance microbienne est beaucoup plus large dans la demi-plaqueensemencée avec la souche B/2 que dans l'autre. Il existe

donc une plus grande sensibilité du germe mutant B/2 à la cystéine.

Par la même méthode, nous avons pu mettre en évidence sa plus grande sensibilité à la tryptaflavine, le rivanol, la mépacrine, le paranitrophénol et la paranitrophénylglycine (Fig. 2).

Si nous utilisons, comme milieu de culture, du bouillon gélosé à 2%, nous observons encore une plus grande sensibilité du mutant B/2 aux substances étudiées mais les anneaux d'inhibition microbienne sont moins larges qu'en milieu synthétique.

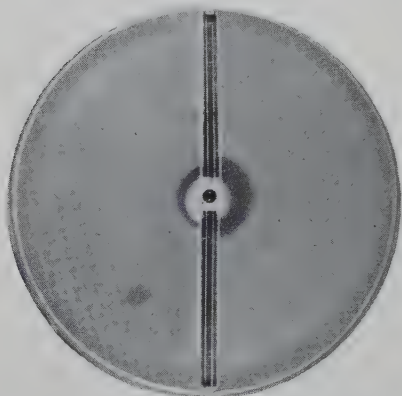


Fig. 2. Plaque de gélose minérale montrant les différences de sensibilité à la tryptaflavine du germe coli B et de son mutant résistant au bactériophage  $T_2$ .

Le demi-plaque de gauche a été ensemencée avec du coli B, celle de droite a reçu du coli B/2.

Le mutant B/2 est réfractaire à l'action du bactériophage  $T_2$ . Nous avons pu déterminer qu'il était plus sensible à l'action de divers agents que le germe normal. Ces résultats plaident en faveur de la première hypothèse émise, qui explique le phénomène du double anneau par l'action directe de la substance étudiée sur le germe résistant. Voyons maintenant si la seconde hypothèse doit être totalement écartée ou si, au contraire, la zone de lyse maximale est due à la fois à une hypersensibilité du mutant B/2 à certains agents chimiques et à une exaltation de la viru-

lence du bactériophage sous l'influence de ce même agent.

**Expérience 7.** Préparons des boîtes de Petri comme pour l'expérience 6, mais ensemencons les demi plaques respectivement avec un mélange de colibacille B/2 et de bactériophage  $T_2$ . Après incubation pendant 24 heures à 37° C. on note que les zones d'inhibition de part et d'autre de la cloison sont les mêmes. Le bactériophage n'intervient donc nullement, par une exaltation éventuelle de sa virulence, dans la production du phénomène.

#### DISCUSSION ET CONCLUSIONS.

L'étude de la cystéine, de la tryptaflavine, du rivanol, de la mépacrine, du paranitrophénol et de la paranitrophénylglycine sur la bactériophagie en milieu synthétique solide montre que, outre

une action anticoli B et antibactériophage  $T_2$ , ces agents exercent à 37° C. un effet se traduisant par la disparition de colonies de coli résistant au phage  $T_2$ . La zone de lyse maximale est due à une plus grande sensibilité des germes mutants B/2 à ces divers agents.

Pour qu'elle apparaisse certaines conditions doivent néanmoins être réalisées: 1) le milieu doit être synthétique; 2) la concentration en bactériophage doit être suffisante; 3) la température doit être voisine de 37° C.

### 1) Le milieu synthétique.

Les diverses substances étudiées (cystéine, certaines acridines, certains dérivés nitrés) diffusent plus aisément dans un milieu simple (solution de divers sels dans de l'eau distillée) que dans un milieu complexe comme la gélose ordinaire (bouillon gélosé) sur les protéines duquel elles peuvent se fixer. En gélose synthétique, on assiste à un étalement des diverses concentrations à partir du godet central; il existe de ce fait une large zone où les substances étudiées exercent une action anticoli résistant sans être antiphage. Ceci permet l'apparition d'un anneau où les germes normaux sont lysés et où les germes résistants ne peuvent se multiplier et donner naissance à des colonies visibles à cause de leur sensibilité particulière. Cet anneau de lyse maximale est compris entre la zone où les concentrations sont suffisantes pour empêcher l'apparition de la lyse mais où les germes sensibles se développent normalement, et la zone périphérique, où les concentrations sont trop faibles pour inhiber la multiplication des germes résistants.

### 2) La concentration en bactériophage.

La concentration en bactériophage doit être suffisante pour que la lyse des germes sensibles soit totale, ce que l'on obtient avec des dilutions faibles ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ) de filtrat sur bougie L III d'une culture de 24 heures en bouillon, de coli sensible additionné de principe lytique. A des dilutions ( $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ), la lyse n'est pas complète, les taches de bactériophage, bien que pouvant être confluentes, dentèlent une nappe microbienne constituée en grande partie par des germes normalement sensibles qui, à cause de leur plus grande résistance à l'agent chimique, masquent la zone de lyse maximale éventuelle.



### 3) La température.

BLOCH (2), dans une étude très complète de l'influence de la température sur le cours de la réaction bactériophagique, rappelle que la multiplication des bactériophages et la lyse ne se produisent qu'entre des limites de température comprises entre 20 et 40° C. Nous avons vu, toutefois, que le bactériophage T<sub>2</sub>, utilisé dans nos expériences, était encore capable de se multiplier à 16° C. et de provoquer l'apparition, sur plaque de gélose, d'un nombre considérable de plages vierges. Celles-ci sont cependant beaucoup moins nombreuses qu'à 37° C. Dès lors, nous pouvons considérer que la température intervient dans la production du double anneau comme un facteur favorisant ou défavorisant la multiplication des particules de bactériophage, une température inférieure à 20° C. empêchant l'apparition du nombre de plages nécessaire à la mise en évidence de la zone de lyse maximale (cfr. influence de la concentration en bactériophage).

La recherche des causes d'apparition de la zone de lyse maximale nous a conduit à l'étude de certaines propriétés remarquables des germes coli B ayant acquis la résistance à l'un des bactériophages de la série T de Delbrück. Les faits observés seront publiés ultérieurement.

### Bibliographie.

1. BERGER, E. et ROESLI, H. 1927. Z. f. Hyg. **107**, 731.
  2. BLOCH, H. 1943. Arch. ges. Virusforsch. **2**, 268.
  3. FLU, P. C. 1926. Centralbl. f. Bakt. I, **97**, 1.
  4. FOSTER, R. A. C. 1948. J. Bact. **56**, 795.
  5. MOLRON, J. H. 1952. Comptes rendus Soc. bel. Biol. **146**, 1263 et 1264.
  6. MUTSAARS, W. 1950. Comptes rendus Soc. bel. Biol. **143**, 1435.
  7. NICOLLE, P. et MIMICA, M. Ann. Inst. Past. **73**, 1072.
  8. SPIZIZEN, J., HAMPIL, B. et KENNEY, J. C. 1951. J. Bact. **62**, 331.
-



(Laboratory of Microbiology, Technological University, Delft, Holland).

## STUDIES ON TRUE DISSIMILATORY NITRATE REDUCTION

### IV. ON ADAPTATION IN *MICROCOCCLUS* *DENITRIFICANS*<sup>1)</sup>

by

A. J. KLUYVER and W. VERHOEVEN

(Received March 23, 1954).

#### INTRODUCTION.

In some preceding publications (KLUYVER, 1953; KLUYVER and VERHOEVEN, 1954; VERHOEVEN, KOSTER and VAN NIEVELT, 1954) the versatility of the potencies of *Micrococcus denitrificans* has been stressed. However, from these studies it has become clear that the metabolic properties of the cells of this bacterium are largely dependent on their previous history.

Already VAN OLDEN (1940) proved with the aid of the "resting cell" technique that only those cells are able to bring about denitrification which originate from cultures in which this process has been the source of energy. So VAN OLDEN concluded that nitratase must be considered an adaptive enzyme.

In the studies cited an analogous situation was encountered with regard to the ability of *M. denitrificans* to consume molecular hydrogen.

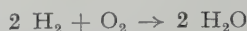
If grown on a medium containing peptone and nitrate the cells were able to bring about a vigorous denitrification with organic hydrogen donators, but unable to consume hydrogen. However, when cultivated in a hydrogen atmosphere in a mineral nitrate containing medium to which minute quantities of organic substances had been added, the cells proved to be able to catalyse not only the reaction:



---

<sup>1)</sup> Part I, II and III: Antonie van Leeuwenhoek **20**, 93, 241, 273, 1954.

but also the "Knallgas" reaction:



The aim of the present study has been to investigate in a general way in how far the preceding cultural conditions influence the behaviour of "resting cells" of *Micrococcus denitrificans* towards the hydrogen donators: organic compounds and molecular hydrogen, and towards the hydrogen acceptors: oxygen, nitrate, nitrite and nitrous oxide. As far as the three latter compounds are concerned, it seemed of special importance to collect additional evidence for the conclusion of KLUYVER and VERHOEVEN (1954) that adaptation to nitrate is accompanied by a simultaneous adaptation to the two other acceptors.

#### EXPERIMENTAL METHODS.

In all experiments use has been made of the manometric method. The bacteria were invariably cultivated on agar media. These media were always inoculated with a relatively heavy suspension of cells derived from a two to three days old stock culture on peptone agar, 1%  $\text{KNO}_3$ , 0.5% glycerol. All experiments were made with a culture of *Micrococcus denitrificans* which had been isolated more than forty years ago by BEIJERINCK (cf. VERHOEVEN, KOSTER and VAN NIEVELT, 1954). In part of the experiments the medium of GROHMANN (1924) containing 0.1%  $\text{Na}_2\text{CO}_3$ , 0.02%  $\text{MgSO}_4$ , 0.1%  $\text{NH}_4\text{Cl}$ , 0.02%  $\text{NaCl}$ , 0.07%  $\text{K}_2\text{HPO}_4$  and a trace of  $\text{FeCl}_3$  was used as a basic medium, to which according to needs certain additions were made. In cases in which heterotrophically grown bacteria were required 1% peptone agar, 1%  $\text{KNO}_3$ , 1% glucose was always used. After 40-48 hours incubation at 30° C. the cells were harvested, washed with a 1/15 mol phosphate buffer pH 7.2, centrifuged, again washed and centrifuged, and resuspended in the buffer.

The main vessel of the Warburg apparatus contained 1.5 ml of the cell suspension, the side bulb was filled with 0.2 ml of the phosphate buffer; in the experiments with nitrate or nitrite a 1% solution of these compounds in the buffer was used. In the experiments with organic donators the buffer also contained 1% of the compound in question.

If the air in the vessels was replaced by hydrogen or nitrogen, the gas was purified in such a way that all traces of oxygen were removed. The nitrous oxide employed was purified by freezing in

liquid air, removal of the non-condensed impurities by pumping, after which the liquid air was taken away and the gaseous nitrous oxide was transferred into a gas burette. If mixtures of gases were employed, the vessels were flushed with at least 1 l of the appropriate gas mixture.

In the experiments with nitrate and nitrite as hydrogen acceptor, and molecular hydrogen as donator, 100%  $H_2$  was used; with organic hydrogen donators the gas atmosphere consisted of 100%  $N_2$ .

In the experiments with nitrous oxide as an acceptor use was made of a gas atmosphere consisting of 10%  $N_2O$ , 90%  $H_2$ .

In the respiration experiments with molecular hydrogen as donator the gas atmosphere consisted of 60%  $H_2$ , 32%  $N_2$  and 8%  $O_2$ ; in case of an organic donator air was used.

As a rule the vessels were allowed to attain temperature equilibrium in the water bath for 10-15 minutes.

## PART I.

### ADAPTATION WITH REGARD TO THE HYDROGEN ACCEPTORS.

#### 1. THE INTERCHANGEABILITY OF OXYGEN AND NITRATE AS A HYDROGEN ACCEPTOR.

On the basis of growth experiments it is generally accepted that denitrifying bacteria can interchange free oxygen and nitrate as hydrogen acceptor. It seemed, however, important to investigate in how far "resting cells" of *M. denitrificans* were under all conditions equipped with the catalytic systems for the use of each of these acceptors. Although VAN OLDEN (1940) had already proved that in the case of organic hydrogen donators the use of nitrate as an acceptor is restricted to cells grown under conditions of obligate denitrification, it seemed worth-while to check this point also in experiments with molecular hydrogen as donator.

Moreover, also a study of the reverse situation seemed desirable, in other words can cells grown on the system hydrogen-nitrate also oxidize hydrogen with oxygen?

These two points have been dealt with in the following experiments.

In the first place *M. denitrificans* was grown under conditions of strict autotrophy, viz., on Grohmann agar in an atmosphere consisting of: 50%  $H_2$ , 40%  $N_2$  and 10%  $O_2$ .

The results of the manometric experiments are reproduced in Fig. 1.

Taking into account the almost negligible intensity of both endogenous respiration and endogenous  $H_2$  uptake, it is clear that the "resting cells" are very active in catalysing the "Knallgas"-reaction. However, if in the "Knallgas"-system the oxygen is replaced by nitrate — and the same appeared to hold for nitrite and nitrous oxide — practically no hydrogen was consumed. In the course of the nitrate and nitrite experiments apparently a slight adaptation took place.

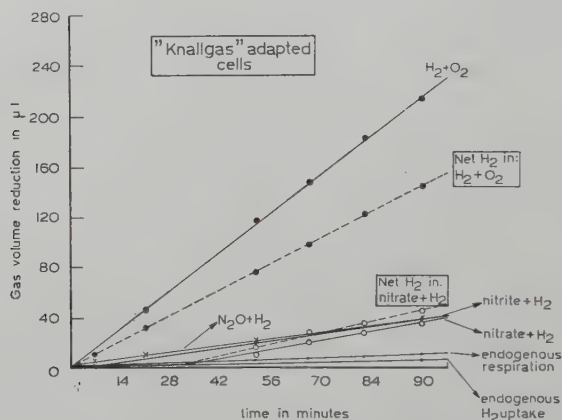


Fig. 1. *Micrococcus denitrificans* grown on Grohmann agar in an atmosphere consisting of 50%  $H_2$ , 40%  $N_2$  and 10%  $O_2$ .

This experiment supports the generally accepted view regarding the adaptive character of the nitratase which is absent in cells grown in a medium lacking nitrate.

Next an experiment was made in which the reverse situation was studied. This time the bacteria were grown in a hydrogen atmosphere on Grohmann agar with addition of 1%  $KNO_3$  and a drop of yeast autolysate <sup>1)</sup>.

The results of the experiment are reproduced in Fig. 2.

At this place only the behaviour towards the system hydrogen/nitrate and hydrogen/oxygen shall be discussed. It appears that although the bacteria had been cultivated in complete absence of free oxygen, they were as well able to consume hydrogen with oxygen as with nitrate. The amounts of hydrogen consumed in

<sup>1)</sup> For the addition of yeast autolysate to the mineral Grohmann medium in this and following experiments the reader is referred to VERHOEVEN, KOSTER and VAN NIEVELT (1954).

either of the two cases have been calculated, and are represented in the figure as dotted lines. In an earlier experiment — cf. VERHOEVEN, KOSTER and VAN NIEVELT (1954) — the rates of hydrogen consumption were found to be equal, suggesting that the capacity of the hydrogenase is the limiting factor in both processes. This time a slight deviation is present, although the rates are still of the same order of magnitude. It is noteworthy that despite the adaptation to nitrate somewhat more hydrogen has been consumed with oxygen than with nitrate.

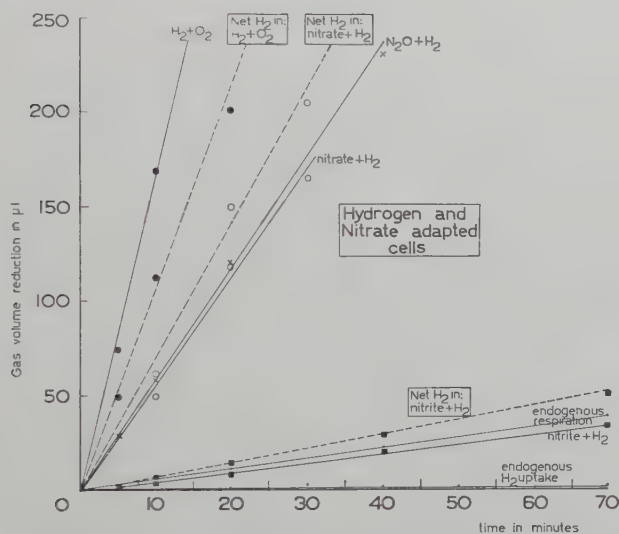


Fig. 2. *Micrococcus denitrificans* grown on Grohmann agar with 1%  $\text{KNO}_3$  and a drop of yeast autolysate in a hydrogen atmosphere.

## 2. SIMULTANEOUS ENZYMATIC ADAPTATION TO THE HYDROGEN ACCEPTORS IN DENITRIFICATION.

Following the lead of SACKS and BARKER (1952), and of ALLEN and VAN NIEL (1952), the authors have in the second publication of this series reported on a few experiments made in order to test whether bacteria adapted to the use of nitrate as hydrogen acceptor were simultaneously adapted to the use of nitrous oxide as such. Negative results of these experiments would have excluded the possibility that nitrous oxide acts as an intermediate in the conversion of nitrate into nitrogen. However, the results obtained were positive,



thus leaving the possibility of the intermediate character of nitrous oxide fully open.

It seemed attractive to collect further data regarding the conditions which determine the adaptive formation of the enzyme systems active in the hydrogenation of the hydrogen acceptors in denitrification.

In this respect it should be mentioned that ALLEN and VAN NIEL (1952) report that nitrate adapted cells of *Pseudomonas stutzeri* were simultaneously adapted to nitrite and nitrous oxide, nitrite adapted cells simultaneously adapted to nitrate and nitrous oxide, but that nitrous oxide adapted cells were only able to use nitrate and nitrite after a lag period.

It was investigated in how far the same would hold for *Micrococcus denitrificans*. Since for this organism the possibility exists to employ molecular hydrogen as hydrogen donator, advantage could be taken of the fact that in this case a manometric determination of nitrous oxide consumption is more reliable than the determination used by the American investigators, in whose experiments the small increase in pressure due to the difference in solubility of nitrous oxide and nitrogen is decisive.

Moreover, also in the case of nitrate and nitrite the use of molecular hydrogen as donator makes it easy to express the pressure changes observed in terms of hydrogen consumed, thus facilitating a mutual comparison.

For these reasons in the following experiments always hydrogen adapted bacteria have been used. In each series an experiment was included in which the activity of the cell suspension towards "Knallgas" was determined.

#### *a. Nitrate adapted bacteria.*

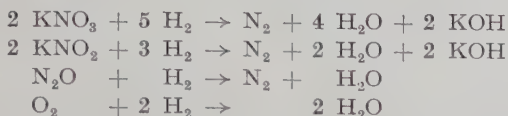
For the behaviour of the nitrate adapted bacteria the reader is referred to the experiment described in Section 1, and reproduced in Fig. 2. As has already been remarked the curve dealing with hydrogen in presence of nitrate clearly shows that there is a high activity of the bacteria in this system. In contrast herewith it is remarkable that under the conditions of the experiment nitrite is an unsatisfactory hydrogen acceptor. This point will be dealt with under *d*.

The gas absorption is again quite considerable in the experiment with a mixture of nitrous oxide and hydrogen.

The question arises whether in the experiments under conside-

ration the hydrogenase is the limiting factor in the gas consumption.

From the equations:



it follows that in these four processes the net hydrogen consumption will respectively be 5/4, 3/2, 1 and 2/3 time the gas consumption observed.

In Fig. 2 the curves for the net hydrogen consumption in nitrate, in nitrite and in oxygen are given as dotted lines; the curve for net hydrogen consumption with nitrous oxide is the same as the curve for gas absorption in  $\text{N}_2\text{O} + \text{H}_2$ . Apparently the hydrogen consumption in the nitrous oxide experiment is somewhat lower than in the nitrate experiment, and the latter is again surpassed by the hydrogen consumption with oxygen. The differences, however, are not very considerable and may be due to the imperfections of the experimental technique. However, a definite conclusion that the hydrogenase capacity controls the reaction rates cannot be drawn. Only in the experiment with nitrite and hydrogen the net hydrogen consumption is obviously markedly reduced.

#### *b. Nitrite adapted bacteria.*

Despite the discouraging experience with nitrite as hydrogen acceptor an attempt was made to grow *Micrococcus denitrificans* on Grohmann agar with nitrite in a hydrogen atmosphere. The growth of the bacteria proved to be very scanty, but still sufficient to make some manometric experiments possible. The results obtained in the experiments with these "nitrite adapted" bacteria are given in Fig. 3.

The most striking result is undoubtedly that the "nitrite adapted" bacteria do not show the slightest activity in the manometric experiment with nitrite as acceptor. We shall return to this question under *d*. However, with nitrate, nitrous oxide and oxygen again a good gas consumption could be observed. A calculation of the net hydrogen consumption in the three cases shows that the bacteria in a nitrous oxide/hydrogen atmosphere are more active than the same bacteria in a nitrate/hydrogen environment, whilst the activity is largest in the "Knallgas" experiment. Evidently in these cases the rate of the acceptor hydrogenation determines the rate of the reaction.

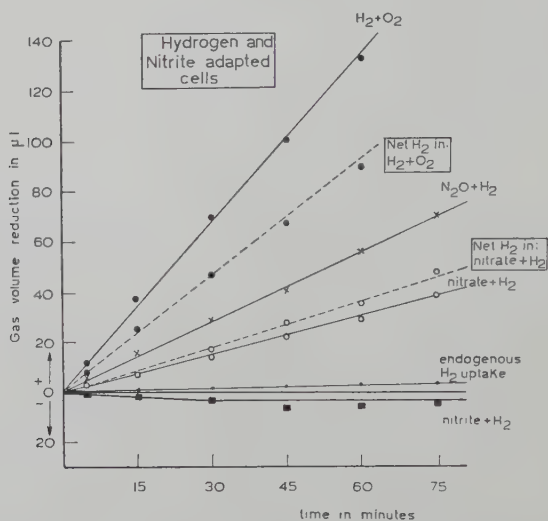


Fig. 3. *Micrococcus denitrificans* grown on Grohmann agar with 0.5%  $\text{KNO}_2$  and a drop of yeast autolysate in a hydrogen atmosphere.

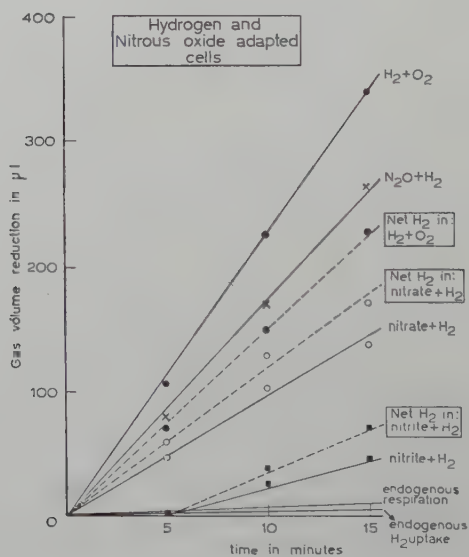


Fig. 4. *Micrococcus denitrificans* grown on Grohmann agar and a drop of yeast autolysate in an atmosphere consisting of 50%  $\text{H}_2$  and 50%  $\text{N}_2\text{O}$ .

### c. Nitrous oxide adapted bacteria.

As has already been shown in 1910 by BEIJERINCK it is possible to grow certain denitrifying bacteria on the simple system molecular hydrogen with nitrous oxide as hydrogen acceptor. It was found that this also holds for *Micrococcus denitrificans*. The bacteria used in the manometric experiments reported in Fig. 4 were grown on Grohmann agar with a drop of yeast autolysate, in contact with a gas mixture consisting of 50%  $N_2O$  and 50%  $H_2$ .

Also in this case the bacteria were quite able to consume large amounts of molecular hydrogen with nitrous oxide, nitrate and oxygen as hydrogen acceptor. With nitrite as acceptor, however, again difficulties were encountered. After a certain lag time gas consumption became perceptible, but its intensity remained low as compared with the gas consumption in the cases of nitrate and nitrous oxide.

In this experiment the calculated net hydrogen consumption with nitrous oxide as acceptor again surpassed the hydrogen consumption in the nitrate experiment, and this time even that in the "Knallgas" experiment. These results point to the occurrence of a specific nitrous oxide hydrogenase in the cells. With this type of bacteria too there is no clear control of the reaction rates by the hydrogenase capacity.

### d. Further experiments regarding the system nitrite/hydrogen.

From the three preceding experiments it is evident that as far as molecular hydrogen is concerned nitrite is a much less suitable hydrogen acceptor than both nitrate and nitrous oxide. This was the more surprising, because this result is at variance with all previous experiences. Only quite recently BAALSRUD and BAALSRUD (1954) have reported an analogous observation for their autotrophic *Thiobacillus denitrificans*.

The exceptional behaviour of nitrite in our experiments asked for some further investigation.

Now it is well known that especially in the acid region nitrite has a toxic action on many cells. It was, therefore, decided to make a number of experiments in slightly alkaline media; phosphate buffers with pH varying between 7.4 and 8.6 were used. The results of this experiment are reported in Fig. 5.

Though the results are somewhat irregular, evidently in this region

the pH has no significant influence. In all cases the difference between the gas consumption by bacteria on nitrite and hydrogen was considerably lower than that on nitrate and hydrogen.

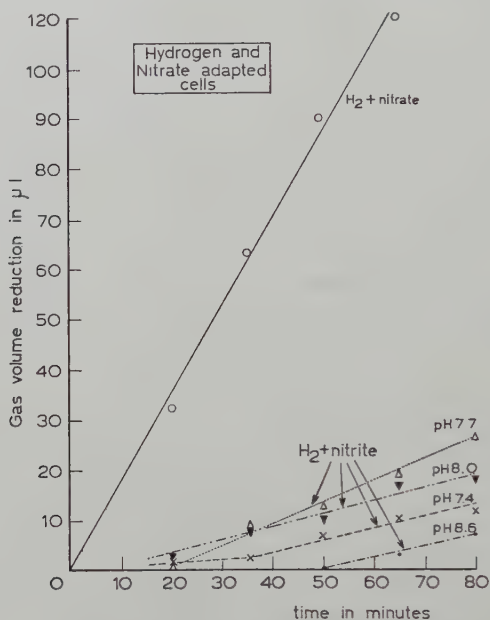


Fig. 5. *Micrococcus denitrificans* grown on Grohmann agar with 1%  $\text{KNO}_3$  and a drop of yeast autolysate in a hydrogen atmosphere.

The circumstance that in earlier studies on nitrite as hydrogen acceptor use had been made of organic hydrogen donors suggested the possibility that the unusual behaviour of nitrite in our experiments would be directly connected with the use of molecular hydrogen. In other words nitrite might have a specific inhibiting action on the catalyst acting on the molecular hydrogen: the hydrogenase.

In order to test this possibility an experiment was first made in which next to nitrate and nitrite also a mixture of both acceptors was used. The results of this experiment are reproduced in Fig. 6.

The interpretation of these results is not easy. On the one hand the simultaneous presence of nitrite in the nitrate containing medium initially brings about a marked inhibition of the nitrate hydrogenation which could be ascribed to a partial inhibition of the hydrogenase by nitrite. But after 5 minutes this inhibition is removed, and from this moment on there is practically no difference



in gas absorption in the presence and in the absence of nitrite. A duplicate experiment yielded the same result.

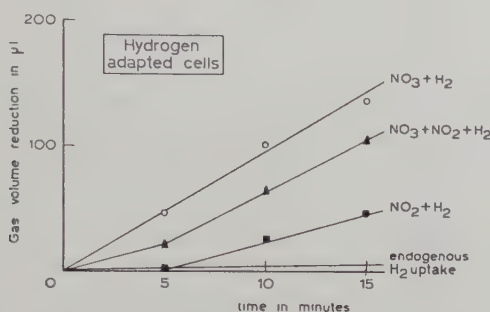


Fig. 6. *Micrococcus denitrificans* grown on Grohmann agar and a drop of yeast autolysate in an atmosphere consisting of 50%  $\text{H}_2$  and 50%  $\text{N}_2\text{O}$ .

In the meantime it remains extremely likely that also in the hydrogenation of nitrate with molecular hydrogen nitrite will act as an intermediate. Obviously under these conditions the nitrite concentration in the cell will be very low, and thus it seemed possible that the high nitrite concentration used in the nitrite-acceptor experiments would be responsible for the hydrogenase inhibition. For this reason it was tested in how far a fourfold reduction of the

TABLE I.

Hydrogen consumption by *Micrococcus denitrificans* in two media containing different nitrite concentrations, compared with that in an analogous medium containing nitrate. Bacteria grown on Grohmann agar with 1%  $\text{KNO}_3$  and a drop of yeast autolysate in a hydrogen atmosphere.

| Time<br>in<br>min. | Gas volume reduction<br>in $\mu\text{l}$ |                       | Net hydrogen<br>consumption in $\mu\text{l}$ |                       | Ratio<br>hydrogen<br>consumption<br>$\text{NO}_3'/\text{NO}_2'$ |
|--------------------|------------------------------------------|-----------------------|----------------------------------------------|-----------------------|-----------------------------------------------------------------|
|                    | 2 mg $\text{NO}_3'$                      | 2 mg $\text{NO}_2'$   | 2 mg $\text{NO}_3'$                          | 2 mg $\text{NO}_2'$   |                                                                 |
| 5                  | 8.2                                      | 0                     | 10.5                                         | 0                     | ∞                                                               |
| 10                 | 48.2                                     | 0                     | 60.2                                         | 0                     | ∞                                                               |
| 20                 | 118.7                                    | 1.3                   | 148.2                                        | 2.0                   | 74                                                              |
| 30                 | 164.1                                    | 2.6                   | 205.0                                        | 3.9                   | 55                                                              |
|                    | 2 mg $\text{NO}_3'$                      | 0.2 mg $\text{NO}_2'$ | 2 mg $\text{NO}_3'$                          | 0.2 mg $\text{NO}_2'$ |                                                                 |
| 5                  | 50.7                                     | 6.9                   | 63.5                                         | 10.3                  | 6.2                                                             |
| 15                 | 164.1                                    | 37.3                  | 205.1                                        | 56.0                  | 3.7                                                             |
| 25                 | 266.1                                    | 67.8                  | 332.6                                        | 101.8                 | 3.3                                                             |
| 40                 | 430.0                                    | 117.3                 | 537.5                                        | 175.4                 | 3.1                                                             |

nitrite concentration would affect the result. In both experiments a control was run in which nitrate was used in the usual concentration. The results are collected in Table I.

Table I shows clearly that also in the experiment with the low nitrite concentration the hydrogen consumption is much less than in the corresponding nitrate experiment. However, the figures leave no doubt that with decreasing nitrite concentration — in the low concentration experiment the nitrite was practically consumed at the end of the experiment — the ratio of the hydrogen consumption in the nitrate and in the nitrite experiment becomes more and more favourable for the nitrite.

Altogether it seems probable that the abnormal behaviour of nitrite as acceptor is due to an inhibiting action of higher nitrite concentrations on the hydrogenase (cf. also under *e*).

*e. General consideration of the question of the simultaneous enzymatic adaptation to the various hydrogen acceptors.*

All experiments reported in the sub-sections *a-d* had as chief aim to get an insight into the question whether an adaptation to one of the hydrogen acceptors suitable for denitrification, *i.e.* nitrate, nitrite and nitrous oxide, automatically leads to an adaptation to the two others. In view of the fact that until now all investigators agree that adaptation to nitrate always involved an adaptation to nitrite as well, the main point of interest in the experiments was to decide whether an analogous relation between nitrate and nitrous oxide would obtain.

If we now first survey the results described in sub-section *a* and *c*, it is clear that we may conclude that the nitrate-adapted bacteria were, indeed, simultaneously adapted to nitrous oxide, and that also the reverse relation holds. As for the first part of this conclusion we need only remark that it leaves open the possibility that nitrous oxide is a normal intermediate in the hydrogenation of nitrate, however, without bringing a decisive proof for this assumption. The second result is the more remarkable, since it could be easily understood, that a cell equipped with a catalyst determining the hydrogenation of nitrous oxide will not necessarily also have at its disposal the catalytic systems required for the hydrogenation of nitrate and nitrite. The experiment reported in Fig. 4, indeed, suggests that the capacity of a specific nitrous oxide hydrogenase surpasses

the capacity of the nitrataase in the nitrous oxide adapted cells.

The abnormal behaviour of nitrite as hydrogen acceptor has already been discussed under *d* and the conclusion has been drawn that probably a specific inhibition of the hydrogenase by nitrite is responsible for the anomaly in question.

In order to give further support to this hypothesis it was decided to make another experiment, in which the molecular hydrogen was replaced by glucose as hydrogen donator. In this experiment both the denitrification with nitrate and with nitrite, and also the respiration were determined for bacteria which had been anaerobically grown on 1% peptone agar, 1%  $\text{KNO}_3$  and 1% glucose, and thus were nitrate adapted. The results are reported in Fig. 7. In this case the curves of the denitrification experiments represent the amount of nitrogen evolved, while the respiration curve gives the oxygen consumption as usual.

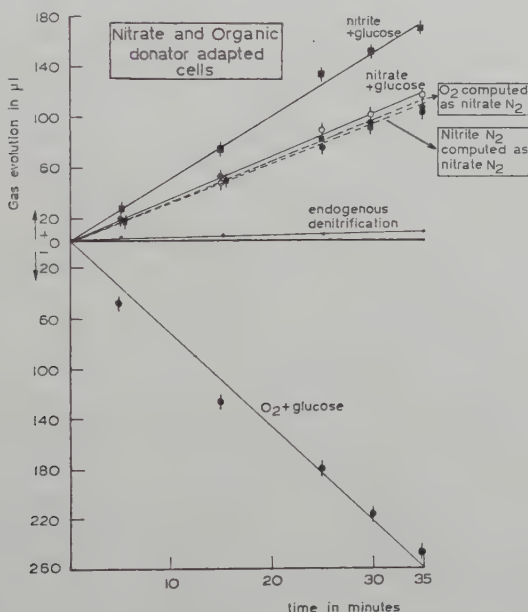
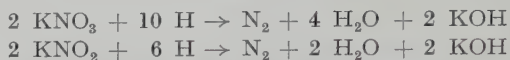


Fig. 7. *Micrococcus denitrificans* grown on peptone agar, 1%  $\text{KNO}_3$ , 1% glucose, in a nitrogen atmosphere.

As far as the experiment with nitrite as hydrogen acceptor is concerned, the result is most gratifying. In the first place there is this time no lag phase in the nitrogen production. Moreover, it is,

of course, possible to calculate the ratio between the nitrogen amounts evolved with nitrate and with nitrite as acceptor on the assumption that the capacity of the glucose dehydrogenase determines the rate of both processes.

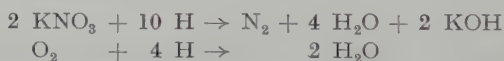


The amount of nitrogen evolved from nitrate should equal 3/5 of the amount of nitrogen evolved from nitrite, if the said assumption is valid. The latter amount is indicated by a dotted line which shows that the said relation indeed holds. This result also gives evidence that in these manometric experiments no  $\text{N}_2\text{O}$  is formed.

In this case there is, therefore, not the slightest objection against the acceptance of nitrite as an intermediate in nitrate reduction, and this result supports the above expressed assumption that the abnormal behaviour of nitrite in the experiments with molecular hydrogen is due to an inhibition of hydrogenase by higher concentrations of nitrite. Since in the experiments of BAALSRUD and BAALSRUD (1954) on *Thiobacillus denitrificans* the nitrite effect also depends on the nature of the hydrogen donator, it seems probable that here too the nitrite acts on the dehydrogenase system, and not on the nitrataase as suggested by the authors.

Finally attention should be given to the respiration experiment which was also included in this series. It is clear that it is possible to check in how far in the respiration experiment too the rate of the process is determined by the capacity of the glucose dehydrogenase.

From the equations:



it is evident that, if the postulated condition holds, the ratio of the amount of oxygen consumed in respiration to the amount of nitrogen evolved in nitrate denitrification should be 4/10. The upper dotted line in Fig. 7 is the result of the conversion of oxygen consumed into nitrogen evolved on the basis of equal hydrogen donation. Again the agreement with the directly observed nitrogen evolution is highly satisfactory.

This result is extremely interesting, because it clearly shows that the always advocated homology between respiration and denitrification even bears a quantitative character.

It should be acknowledged that, as discussed earlier, in the experiments with molecular hydrogen results were obtained which did not completely agree with the assumption that here too the rate of the reactions with the various hydrogen acceptors was fully determined by the hydrogenase capacity. Leaving the abnormal behaviour of the nitrite apart, it can be said that the net hydrogen consumption with  $O_2$ , nitrate and nitrous oxide were of the same order of magnitude, but did not quite coincide. It seems possible that in this respect the fact that the hydrogen donator can only enter into the cell in a low concentration, *viz.*, as dissolved hydrogen, affects the results.

## PART II.

### ADAPTATION WITH REGARD TO THE HYDROGEN DONATORS.

#### 1. The interchangeability of lactate and molecular hydrogen as hydrogen donator.

Already NIKLEWSKI (1914) observed that addition of some organic substrate to a growing culture of his *Hydrogenomonas vitrea* promoted the growth, but at the same time inhibited the oxidation of hydrogen in these cultures. Also RUHLAND (1924) in his fundamental study on the "Knallgas-bakterien" reports the same experience.

In their "resting cell" experiments with the hydrogen oxidizing bacterium *Hydrogenomonas flava* KLUYVER and MANTEN (1942) established in the first place that the oxidation of hydrogen only takes place, when the bacteria had been grown in a "Knallgas"-atmosphere. Suspensions of cells grown under heterotrophic conditions were unable to bring about any hydrogen oxidation, but respired normally in the presence of organic substrates. On the other hand the autotrophically grown bacteria proved to be able to bring about also the heterotrophic type of oxidation. However, in contrast to what might be expected from the observations by NIKLEWSKI and RUHLAND it was found that on simultaneous addition of hydrogen and an organic compound both substrates were oxidized; even the rates of these oxidations were practically unaffected. This strongly suggests that hydrogen oxidation asks for the presence of a special catalytic system which is different from the dehydrogenase active in normal respiration.

In later years SCHATZ and BOVELL (1952) have reported that the



hydrogen oxidizing properties of "resting cells" of their *Hydrogenomonas facilis* were independent of the preceding cultural history. This observation could, however, not be confirmed by WILSON, STOUT, POWELSON and KOFFLER (1953), although these authors worked with the same strain as used by SCHATZ and BOVELL. However, WILSON *et al.* succeeded in obtaining hydrogen oxidizing cells from heterotrophic cultures by lowering the oxygen tension in these cultures.

It seemed of importance to investigate the conditions determining hydrogenase production also for *Micrococcus denitrificans*. On the other hand the behaviour of autotrophically grown cells towards organic hydrogen donors was also worthy of investigation. In this connection it may be remarked that both KLUYVER and MANTEN (1942), and WILSON *et al.* (1953) found that the ability to dehydrogenate an organic substrate persists during autotrophic growth.

#### a. Glucose adapted bacteria.

It was decided to investigate first the behaviour of bacteria grown in a hydrogen atmosphere on a medium rich in organic matter,

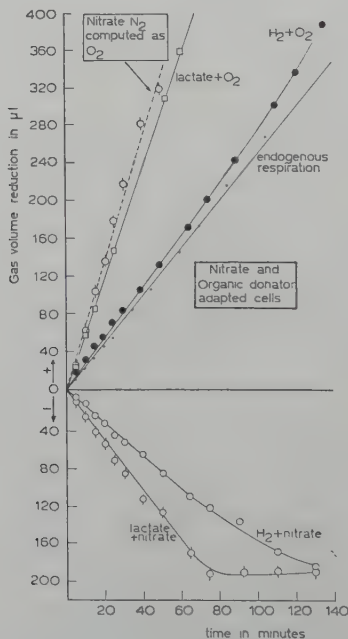


Fig. 8. *Micrococcus denitrificans* grown on peptone agar, 1%  $\text{KNO}_3$ , 1% glucose, in a hydrogen atmosphere.

including glucose. The contact with hydrogen seemed to offer also the possibility of adaptation to this donator.

The medium consisted of peptone agar, 1%  $\text{KNO}_3$ , 1% glucose. In the "resting cell" experiments, the results of which are reproduced in Fig. 8, lactate was used as organic donator.

According to expectation the bacteria bring about a vigorous denitrification with lactate as hydrogen donator. In the aerobic experiment there is also a satisfactory oxidation of lactate.

The endogenous respiration is very considerable, and taking this into account it seems justified to conclude that the "Knallgas"-activity is almost nil, although there is a clear indication of a slight adaptation during the experiment.

The curve dealing with hydrogen in the presence of nitrate remarkably shows a gas production instead of the consumption which would have occurred in the case of denitrification with molecular hydrogen. Apparently we are dealing here with endogenous denitrification, *i.e.*, denitrification at the expense of the reserve compounds present in the cells.

It is worth-while to note again that the rates of both respiration and denitrification are determined by the capacity of the lactate dehydrogenase. If we apply on the figures of lactate denitrification the computation as applied in Fig. 7 we arrive at the dotted line which nearly coincides with the respiration curve.

These observations bring a confirmation of the results obtained by the earlier cited authors for the "Knallgas-bakterien", together with an extension of these results to the case of denitrification with molecular hydrogen.

Evidently hydrogenase production by the bacteria is completely inhibited in the presence of readily available organic hydrogen donators.

#### *b. Hydrogen adapted bacteria.*

As already mentioned in the introductory remarks to this section it has been generally observed that autotrophically grown hydrogen oxidizing cells are able to oxidize also organic substrates. This point was also investigated for *Micrococcus denitrificans*, and at the same time it was tested whether an analogous situation would hold for denitrification. The bacteria were grown on Grohmann agar with a drop of yeast autolysate and 1%  $\text{KNO}_3$  in a hydrogen atmosphere. The results are produced in Fig. 9.

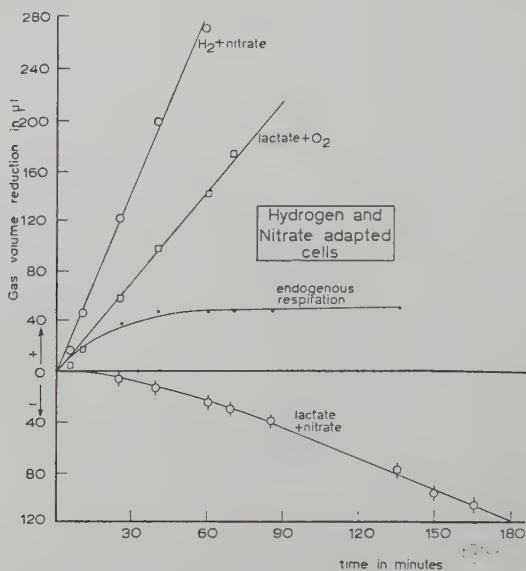


Fig. 9. *Micrococcus denitrificans* grown on Grohmann agar with 1%  $\text{KNO}_3$  and a drop of yeast autolysate in a hydrogen atmosphere.

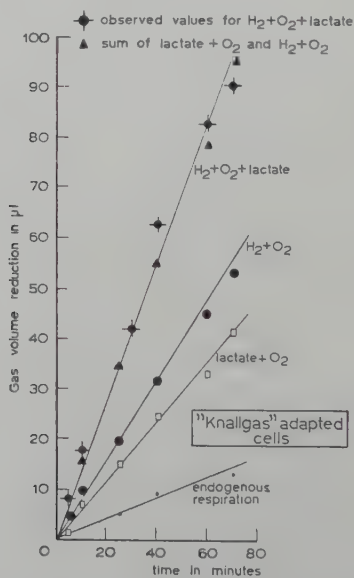


Fig. 10. *Micrococcus denitrificans* grown on Grohmann agar in an atmosphere consisting of 50%  $\text{H}_2$ , 40%  $\text{N}_2$  and 10%  $\text{O}_2$ .

It should be noted in the first place that, as might be expected, "autotrophically" grown bacteria vigorously consume hydrogen in the presence of nitrate. It is, however, surprising that these nitrate adapted bacteria with such an active denitrifying ability in the presence of hydrogen initially are quite unable to use lactate as hydrogen donator in denitrification. Only after a considerable lag period an adaptation manifests itself, but in the course of the experiment the denitrification rate remains low.

In contrast herewith the aerobic dehydrogenation of the lactate proceeds with a satisfactory rate, and without any noticeable adaptation. The only way to explain the different behaviour between the denitrification and the respiration seems to be that the production of lactate dehydrogenase occurs much more rapidly with oxygen than with nitrate as hydrogen acceptor.

In a final experiment it was tested whether the additional effect of hydrogen and lactate oxidation, as observed for *H. flava* by KLUYVER and MANTEN, as also for *H. facilis* by WILSON *et al.*, did also hold for *Micrococcus denitrificans*. The bacteria were grown on Grohmann medium in a "Knallgas" atmosphere. The results of the experiment are reproduced in Fig. 10.

A glance at Fig. 10 suffices to answer this question in a positive sense. From the experiments in which only one hydrogen donator had been added it appears that both the molecular hydrogen and the lactate were actively oxidized. It is very striking that, indeed, the gas consumption observed in the experiment in which both donators had been added simultaneously, practically coincides with the sum of the gas consumptions observed with each of the donators separately. This again clearly demonstrates that in *Micrococcus denitrificans* hydrogen activation and lactate activation are fully independent processes.

#### GENERAL CONCLUSIONS.

The foregoing experimental results lead to the following general conclusions.

Cells of *Micrococcus denitrificans* grown under heterotrophic conditions naturally are able to bring about a dehydrogenation of organic substrates with free oxygen. In order to do the same with nitrate as hydrogen acceptor it is not sufficient that the cells are grown in a medium containing nitrate. As has already been shown by VAN

OLDEN the denitrifying ability is only present in those cells which were grown under such conditions that denitrification acted as the energy yielding process. This means obviously that the nitrate activating catalyst, the nitratase, is an adaptive enzyme, but that its formation is bound to special conditions.

This holds also for the denitrifying ability of the "resting cells" obtained from "autotrophic" cultures.

Heterotrophically grown cells lack the ability to use molecular hydrogen as donator both in their respiration process and in denitrification. This statement holds independently of the question whether hydrogen was present or absent in the gas atmosphere in contact with the culture.

It was, however, shown that cells of *Micrococcus denitrificans* are fully able to oxidize hydrogen with oxygen or with nitrate — provided that nitrate-adapted cells are used — if the cells result from a culture grown under autotrophic conditions with molecular hydrogen as donator. This proves that also the hydrogen activating catalyst, the hydrogenase, is a typical adaptive enzyme.

From the foregoing it should be concluded that in order to obtain an adaptive formation of hydrogenase the mere presence of hydrogen in the atmosphere in contact with the culture medium is not sufficient, but that only those cells which for their growth are bound to the use of hydrogen proceed to the production of hydrogenase.

In this respect there is a remarkable similarity in the processes of nitrate and hydrogen adaptation.

In contrast to these findings is the behaviour of the cells of *Micrococcus denitrificans* towards free oxygen. It was namely found that cells grown under anaerobic conditions, either in a medium with organic substrate and nitrate, or at the expense of hydrogen and nitrate, are always able to oxidize the respective substrates with free oxygen without any sign of adaptation.

Whilst heterotrophically grown "resting cells" can not attack hydrogen, or only slightly after adaptation, autotrophically grown cells dehydrogenate organic compounds with free oxygen without any noticeable adaptation. However, when nitrate is used as acceptor there is an unmistakable adaptation process. In the presence of free oxygen the cells probably produce lactate dehydrogenase very rapidly, whilst this proceeds much more slowly in the anaerobic nitrate medium, although it remains possible that some other phenomenon is involved.



The autotrophic cells grown at the expense of hydrogen oxidize a mixture of lactate and hydrogen at a rate equal to the sum of the rates at which each of the separate compounds are oxidized. This means that the hydrogenase and the organic substrate dehydrogenase act quite independently, which result confirms a similar observation by KLUYVER and MANTEN for *Hydrogenomonas flava*.

Finally it should be remarked that the metabolism of *Micrococcus denitrificans* offers an ideal documentation of the transhydrogenation character of respiration.

Denitrifying bacteria in general are the only organisms for which free oxygen can be replaced by another hydrogen acceptor, in casu: nitrate, without interfering in the least with the normal metabolic activities of the cells, proliferation included.

These bacteria can either oxidize glucose with free oxygen according to the over-all equation:

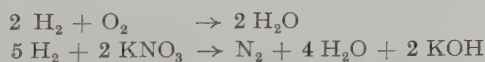


or with nitrate as hydrogen acceptor according to the over-all equation:



In this paper proof has been given that at least under certain conditions this homology even bears a quantitative character.

In addition to this *Micrococcus denitrificans* has the special merit that it makes the transhydrogenation character of its dissimilatory processes almost tangible, because it has the property, unique amongst denitrifying bacteria, to be able to substitute the organic hydrogen donator both in respiration and denitrification by molecular hydrogen. In doing so it brings about the following over-all conversions:



Here too under certain conditions the hydrogen consumption proceeds with the same rate with either oxygen or nitrate.

### S u m m a r y.

From experiments with "resting cells" of a for more than forty years heterotrophically grown culture of *Micrococcus denitrificans* the following conclusions can be derived:

1. Nitratase, the nitrate reducing catalyst, is an adaptive enzyme

which is only present in cells grown in media in which active denitrification has occurred. In this denitrification process nitrate can be replaced by nitrite or by nitrous oxide.

2. Hydrogenase, the catalyst activating molecular hydrogen, is an adaptive enzyme which is only present in cells grown in a medium in contact with a gas atmosphere containing hydrogen, and in so far as this gas is the only available hydrogen donator.

3. Hydrogenase adapted cells, either derived from an autotrophic "Knallgas" culture, or from a culture depending on the nitrate/hydrogen system, are always able to bring about a "Knallgas" reaction; only the second category can also reduce nitrate with molecular hydrogen (nitratase!).

4. Cells grown autotrophically — at the expense of hydrogen — are able to oxidize organic compounds without noticeable adaptation. In the simultaneous presence of hydrogen and an organic compound both substrates are oxidized independently.

5. The oxidation of glucose with either free oxygen or with nitrate proceeds at the same rate, and this holds also for the oxidation of hydrogen. These results present a clear demonstration of the homology of respiration and denitrification.

## References.

- ALLEN, M. B. and VAN NIEL, C. B. 1952. *J. Bact.* **64**, 397.  
BAALSRUD, K. and BAALSRUD, K. S. 1954. *Arch. Mikrobiol.* **20**, 34.  
BEIJERINCK, M. W. mit Mitwirkung von MINKMAN, D. C. J. 1910. *Centralbl. f. Bakt.* **II**, **25**, 30.  
KLUYVER, A. J. and MANTEN, A. 1942. *Antonie van Leeuwenhoek* **8**, 71.  
KLUYVER, A. J. 1953. Some aspects of nitrate reduction. In: *Symposium Microbial Metabolism*. Roma.  
KLUYVER, A. J. and VERHOEVEN, W. 1954. *Antonie van Leeuwenhoek* **20**, 241.  
NIKLEWSKI, B. 1914. *Centralbl. f. Bakt.* **II**, **40**, 430.  
VAN OLDEN, E. 1940. *Proc. Kon. Ned. Ak. v. Wet.* **43**, 635.  
RUHLAND, W. 1924. *Jahrb. f. wiss. Bot.* **63**, 321.  
SACKS, L. E. and BARKER, H. A. 1952. *J. Bact.* **64**, 247.  
SCHATZ, A. and BOVELL Jr., C. 1952. *J. Bact.* **63**, 87.  
VERHOEVEN, W. 1952. *Aerobic sporeforming nitrate reducing bacteria*. Thesis, Delft.  
VERHOEVEN, W., KOSTER, A. L. and VAN NIEVELT, M. C. A. 1954. *Antonie van Leeuwenhoek* **20**, 273.  
WILSON, E., STOUT, H. A., POWELSON, D. and KOFFLER, H. 1953. *J. Bact.* **65**, 283.

(Laboratoire de Microbiologie, Institut Agronomique de l'Etat, Gembloux, Belgique).

## THE EFFECTS OF INDUCED SOIL AGGREGATION ON THE OCCURRENCE OF *AZOTOBACTER* AND *STREPTOMYCES*

by

**F. W. HELY and CH. BONNIER**

(Reçu le 5 Mars 1954).

It seems reasonable to expect that any substance which produces increased soil aggregation and therefore greater aeration would tend to favour the development of such strongly aerobic species as those of *Azotobacter* and *Streptomyces*. This, of course, would be providing that adequate nutrients were present in the soil to permit such increased microbial activity and also that the soil-aggregating agents involved were not in some manner toxic or capable of giving rise to toxic decomposition products. The possibility of increased development of these species must be related in addition to the activity of other soil micro-organisms which also may be stimulated by soil aggregation and have a pronounced inhibitory effect on *Azotobacter*, for example, by locking up nutrients and/or producing antibiotics.

Following the studies made with soil fungi by BONNIER and HELY (1953) and with gum-producing bacteria by HELY and BONNIER (1953), investigations were made with the object of determining whether the effects of certain new synthetic soil-aggregating substances might be generally stimulating, depressing or toxic in their end effects on the naturally-occurring *Azotobacter chroococcum* and species of *Streptomyces*. In general, attempts were made to study what actually happened to the populations of these organisms in a loess-derived loam soil, which had just grown a crop of wheat, as the normal and over-all result of applications of soil-aggregating synthetic products.

It could be shown readily at this laboratory that when average,

fertile, lighter-coloured farm soils, not well provided naturally with water-stable macro-aggregates, were taken from the field in late summer, passed through a 1 mm screen, air-dried, aggregated with from 0.1% to 0.3% of active constituent of any of these products, excepting normal "Aglusol", abundant carbohydrate was supplied to them by the addition of 5% of mannitol, and such mixtures were kept at the moist, crumbly stage and incubated at 27° C. for a week, great effects were produced on the *Azotobacter* population. The untreated soils always showed a little darkening after one week of incubation with mannitol but the strongly aggregated soils invariably became quite black at the visible surfaces as though they had become rich in substances of a humic nature and/or there had been an appreciable production of a melanin-like pigment such as that studied by WANG (1951). Scrapings made from the really blackened soil aggregates showed the presence of great numbers of *Azotobacter* cells. Noticeable effects could be obtained by the use of as little as 0.5% of mannitol with highly aggregated soils. No apparent darkening occurred in any soil lot without the addition of mannitol. To observe this effect was easy but its measurement posed a problem.

Also with *Streptomyces* it was readily possible to incubate a series of aggregated moist fertile soils without addition of carbohydrate or with a small amount of soluble starch for a week or more and to obtain from such soils a pronounced aroma characteristic of *Streptomyces*. This effect seemed to be much greater in the case of the well aggregated soils than with the poorly aggregated and untreated soils.

Attempts were made therefore to measure such apparent early effects of the soil-aggregating treatments by precise techniques and to follow through to see to what extent they were maintained.

## 1. MATERIALS AND METHODS.

Two of the synthetic products employed were those used by HANOTIAUX and MANIL (1952), namely the original form of "Krilium" powder as supplied by the "Monsanto" firm and a 5% solution of polyelectrolytic resin known as S.R.S. 88/52 which was free from ammonia and was provided by the Union Chimique Belge for experimental purposes. In addition the commercial product "Aglusol", a 5% solution of active principle as manufactured by the Union Chimique Belge and containing as much as 7 mg per ml

of by-product ammonia was employed, as was also this product from which the ammonia had been removed.

In the *Azotobacter* investigations some use was made of the virtually nitrogen-free and sterile loess material described by MANIL (1952) as Cut No. 1, Briqueterie Laubain upper deposit. The particle size distribution of this material as determined by standard mechanical analyses was 20.1% clay, 20.8% silt, 52.2% very fine sand, 6.7% of fine sand and a small fraction of coarse sand.

In general, however, investigations were made on the local topsoil derived from similar loess material. This contained approximately 14% of clay, 32% of silt, 47% of very fine sand, 4% of fine sand and 3% of coarse sand and colluvial or detrital material. For the main experiments this was taken by random sampling from a field which had just grown a heavy crop of wheat at a time when, with the season's yield of plant residues becoming available in the soil, the *Azotobacter* population was relatively high and should have been near its maximum.

The technique of incubating various petri dish and beaker samples of treated soil material was used considerably. Also replicated pot experiments with treated and untreated soils were set up; in these the level of available  $P_2O_5$  was brought to 0.2% and that of  $K_2O$  to 0.1%. Lucerne seedlings were grown in these pots under favourable soil moisture conditions and in from thin to dense stand for three months under partially controlled autumn glasshouse conditions which resulted in fairly good plant growth.

In the *Azotobacter* investigations four possible methods of measurement of activity and numbers were tried, namely: (1) Kjeldahl determination of total soil nitrogen; (2) pin-point placing of one hundred random very small samples of the finely powdered soil under investigation on to a standard petri dish of nitrogen-free agar medium for *Azotobacter*; (3) preparation of soil plaques in petri dishes of 9 cm diameter, using calcium carbonate where necessary to buffer against acidity and from 0.5% to 5% of mannitol as the energy source, then incubating in a saturated atmosphere at 27° C. for two to three days and (4) soaking of soil samples followed by dilution and plating at various concentrations, using mostly thin layers of nitrogen-free agar in flat-bottomed petri dishes.

The agar medium which was normally employed in dilution plating was a form of Ashby's agar. Under our conditions the 0.1% sodium benzoate medium recommended by HUSSAIN ALEEM (1953) was



evidently not advantageous. Although it made counting of the *Azotobacter* colonies somewhat easier, the typical colonies of *A. chroococcum* developed much faster on the N-free mannitol and the mannitol and glucose agars and the numbers of colonies were never smaller and commonly were greater than on the benzoate agar.

The technique of surface inoculation of the agar with measured drops from a dilution series, as used by MILES and MISRA (1938) and described in more detail by POMALES-LEBRÓN and FERNÁNDEZ (1952), is well adapted to *Azotobacter* studies since virtually pure culture conditions obtain with them. Apart from the savings in materials made possible by this method, it has appreciable advantages statistically. A suitable dilution gives twenty to thirty colonies per drop. The method was used successfully with both N-free mannitol agar and sodium benzoate agar but small colonies of *Azotobacter* are easier to count when the latter medium is employed.

In the studies with *Streptomyces* the method of soaking the soil, diluting and plating was used only in the absence of any suitable technique quite specific for these organisms. Although not a good method for micro-organisms which may produce spores abundantly, and whose mycelium also may fragment somewhat, this was considered to give sufficiently comparative results even though counts resulting from a dilution and plating technique cannot be satisfactory from a quantitative point of view. Various media low in the available carbohydrate source were tested; in general there were so many bacteria present in such soils and so many species of these bacteria were able to grow reasonably well on quite low concentrations of nutrients that the slower-growing *Streptomyces* were greatly inhibited and their colonies normally were either obscured or at least made very difficult to count. To keep down the size of bacterial colonies it was found necessary to pour only very thin layers of agar into really flat-bottomed petri dishes and then to incubate these dishes in the upright position to achieve a fair degree of desiccation of the medium. Best results were obtained by using 6-8 ml per standard-sized dish of an agar identical with Czapek's agar except for the substitution of 2.0 g of soluble starch per l of medium of the sucrose recommended. The reaction of this medium was made neutral and incubation was at 27° C. for one week. The general principle followed was that *Streptomyces* material is favoured as the bacterial colony growth is depressed when the carbohydrate source is in low concentration and of a type not

readily available, the medium is relatively dry and proteins or protein derivatives, such as peptone or tryptone, are excluded.

## 2. RESULTS AND DISCUSSION.

One method for measuring the amount of increased *Azotobacter* activity in a soil originally poor in available nitrogen might be to analyse for total nitrogen content the untreated and the various aggregated soil lots after addition of mannitol and incubation in a moderately moist condition for suitable periods of time. Where pure culture methods are not practicable, as in this case, denitrification may be an important factor. However, we were interested only in the total effect of aggregation of the soil on the possible nitrogen fixation by *Azotobacter* in the presence of adequate amounts of available carbohydrate. It was found that the Kjeldahl method as known to us was not quite precise enough to allow significant differences to be shown in total nitrogen between such well aggregated and untreated or slightly aggregated soils even when a reasonable number of replicates were used.

Another technique which received considerable attention was that of powdering the quickly air-dried soil samples carefully and finely so that they would pass through a 0.25 mm screen and then taking at random from such lots 100 small sub-samples with a fine platinum needle for point inoculation on to a petri dish of N-free agar. In the case of the loess-derived top soil collected from a wheat field soon after harvest, there was normally so much *Azotobacter* present that 100% of colonies or nearly that many were always obtained on such plates from all soil lots. This was especially the case with these soils, even those not treated with aggregating substances, when mannitol had been added. It was not possible to overcome this difficulty by refinement of technique, the method generally proving too gross to show significant differences although the numbers of *Azotobacter* cells may have been much greater in certain soil lots than in others. One fact which was shown consistently by this technique was an appreciable reduction in the number of *Azotobacter* colonies obtained from soil lots treated with normal "Aglusol" but having got little or no additional carbohydrate. This was noticeable for about the first month after such treatment when the soil was placed in pots of various sizes, kept moist at about 80% of the field capacity and held at around 20° C. Such

a result is understandable on the basis of the large amount of ammonium nitrogen introduced to the soil by this product; presumably addition of such available nitrogen would cause a rapid and appreciable maintained reduction in available carbohydrate and other nutrient materials by stimulating the growth of many faster-developing other soil micro-organisms. The relatively slowly growing *Azotobacter* has an advantage over the other members of the microflora only in a soil of high and preferably increasing carbon status and comparatively low content of available nitrogen. The depression of *Azotobacter* was found by this method to be great even when as little as 0.01% of normal "Aglusol" had been used. In view of the large amount of *Azotobacter* in the untreated soil, it seems very unlikely that the nitrogen-depressing effect was confounded here with the difficulties in technique introduced by soil aggregation.

Under the conditions obtaining the *Azotobacter* numbers apparently were at a minimum in these soils during the third and fourth weeks of pot experiments, irrespective of the nature of the soil aggregating substance used and whether the soil supported no plants or a thin or a dense stand of lucerne. During that period 100-point inoculations of undiluted potted soil from lots taken from the above-mentioned wheat field gave fewer than one hundred colonies on most plates and the percentages for control pots ranged from sixty to seventy. At this time numerous pure soil platings made from pots in which there was but little plant growth indicated a consistent trend towards a small actual reduction in *Azotobacter* colony numbers as the result of induced aggregation by the three products which did not contain appreciable available nitrogen. This seemed to be quite reasonable because other studies, such as those of HELY and BONNIER (1953), had revealed that a considerable increase in other microbiological activity had occurred in such aggregated soils; therefore the nutrients available to *Azotobacter* at this time, before any appreciable plant growth had occurred, presumably were at a minimum in the well aggregated soil lots.

Continuing further with this technique, attempts were often made to use the method of diluting the soil samples under investigation with similarly powdered, sterile loess parental material immediately before plate inoculation. Reduction in the number of colonies obtained from one hundred inoculations could be readily obtained by sufficient dilution but significant increases due to aggregation treatments could not be shown, even by using up to five replications,

because adequate dispersion of the top soil and good mixing were not practicable. It was observed commonly that colonies which resulted from point inoculations from well aggregated soils tended to appear earlier and grow faster than those derived from control and poorly aggregated soils, indicating possibly the presence of a greater amount of *Azotobacter* as well as a larger average soil particle size in the former. It seemed that it was not possible to break well aggregated soil lots finely enough to get them mixed with the loess diluent as thoroughly as the untreated soil could be mixed. Microscopic examination showed that the bias was such that the possibility of picking with the needle some original surface soil from any of the dilution mixtures tried was clearly greatest in the case of the control mixture; conversely the chances were least with the powdered strongly aggregated soil lots.

With respect to the soil plaque technique it was found to be quite unsatisfactory to make plaques from any of the loess soil lots taken freshly from the field; so many colonies of *Azotobacter* developed on the surface following the addition of the standard amounts of lime and mannitol that it was quite impracticable to count them in their very young stages and as they developed further they rapidly coalesced. In general, however, suitable numbers of colonies could be obtained from such soils by diluting them with four times their air-dried weight of finely powdered loess parental material before making the plaques. In such cases it was important to add to the mixtures enough calcium carbonate to ensure neutrality and available sources of phosphorus and potassium because of the slightly acid nature and poor nutrient status of the diluent. In this manner it was possible to obtain better mixing of the soil, a much smoother plaque surface, particularly with the well aggregated soils, and good, discrete colony growth in most instances.

Here again significantly greater numbers of *Azotobacter* colonies were seldom obtained from the well aggregated soil lots as compared with the control and weakly aggregated ones, even when they were incubated with various amounts of mannitol. In all likelihood the general problem of aggregation, with the impossibility of dispersing an equal weight of aggregated soil through the diluent nearly as thoroughly as the control soil could be dispersed, made it possible for the occurrence of even appreciably greater numbers of *Azotobacter* cells to be hidden in the case of well aggregated soils. Where mannitol was added in suitable quantity it seemed clear that the



particles of soil derived from well aggregated lots carried more *Azotobacter* than those derived from the control and slightly aggregated lots because the former always produced much larger individual colonies on the plaque surfaces and such differences in colony development were much greater in proportion than the observed differences in particle sizes.

An important point emphasized by these investigations with soil plaques was that the well aggregated soil lots required considerably more mannitol than the comparable control and weakly aggregated samples if anything like comparable growth in height of colonies was to be obtained. Thus it was observed in one series provided with 0.1% of mannitol that the *Azotobacter* colonies were small, persistently moist for several days, symmetrical and noticeably raised in the control lots; by contrast they were each invariably covering a much greater area, but soon drying, rough-surfaced and flat in the case of the moderately aggregated lots and virtually or even completely absent from many samples which had been treated previously with 0.1% or more of polyelectrolyte. If microbiological activity generally is greater in well aggregated soils, it is only to be expected that less of any uniform amount of mannitol added would be available to *Azotobacter* in these well aggregated soils than in control and poorly aggregated lots. The amount of microbiological activity varies from time to time with different soil aggregating treatments; it is no advantage to calculate by experiment, just prior to any major sampling and in such a manner as to avoid bias, the percentage of mannitol required by each soil treatment series to give the *Azotobacter* colonies developing on them access to a same or even approximately same level of mannitol. Therefore no attempt was made to harvest the total colony growth on the surface of each plaque and then to treat the yields obtained as definite measurements of the *Azotobacter* potential of each soil. This was suggested as a possibility in certain special cases by the work of BRAVO (1952) but the chances of using such a method successfully when soil lots of distinctly different aggregation levels are being compared seemed quite remote.

Finally, with respect to the use of the common technique of taking random samples of soil, sub-sampling, soaking, diluting and plating a replicated series in agar or on agar the same problems resulting from differences in soil aggregation were met with in these *Azotobacter* investigations as in the studies dealing with gumproducing



bacteria, reported by HELY and BONNIER (1953). Attempts were made to offset this effect by grinding the soil samples to various degrees of fineness in an agate mortar, by mixing the suspensions vigorously in an electrically-operated blender and by various soaking treatments without shaking; no real success was obtained with either of the first two methods. Although *Azotobacter* is rather less sensitive to air-drying than many common species of soil bacteria it was preferable to take a somewhat moist sample of soil for soaking and a larger lot by the same sub-sampling technique for drying so that the water content could be calculated and an appropriate correction made on the weight of sample investigated.

TABLE 1.

| Time of<br>soaking<br>Hours | Colonies of <i>Azotobacter chroococcum</i> per g<br>of soil |                                      |
|-----------------------------|-------------------------------------------------------------|--------------------------------------|
|                             | Untreated Soil                                              | Aggregated<br>with 0.1% S.R.S. 88/52 |
| 1                           | 568.000                                                     | 148.000                              |
| 2                           | 660.000                                                     | 296.000                              |
| 4                           | 968.000                                                     | 976.000                              |
| 6                           | 1.296.000                                                   | 1.124.000                            |
| 8                           | 1.092.000                                                   | 1.328.000                            |
| 24                          | 780.000                                                     | 980.000                              |
| 48                          | 1.388.000                                                   | 840.000                              |

Table 1 shows typical results obtained from soaking, without shaking, fairly finely broken random soil samples of distinctly different aggregation status from pots in which lucerne had been growing for five weeks. As before 0.5 g sub-samples were soaked in 10 ml lots of sterile water for the times noted above and then the dilution and replication followed, the temperature in each case being 27° C. In the control soil there was only about 14% of water-stable aggregates larger than 0.5 mm in diameter but it seemed that about six hours were required for the cell clumps to break up reasonably when the samples were soaked without shaking. By contrast the treated soil contained about 60% of water-stable aggregates of this size so that here more time was obviously required both for cell clumps to break down and for cells to be freed from aggregates. As the result of experiments of this nature it was decided

that the best compromise was to soak all soil samples for six hours before dilution and plating. The possibility of accelerating this process by an appropriate standard of moderate shaking of the soil and water mixtures was not investigated fully.

Great increases in *Azotobacter* numbers, in terms of colonies on plates, were shown in the artificially aggregated soils of a pot experiment, with eight replications, which had been in progress one month, where the pots carried rather dense growths of lucerne seedlings and the value of stable aggregation in respect to aeration had been emphasized by applying the necessary water from above in a manner simulating heavy rain. The control soil had become compacted and rather sealed at the surface under this treatment and the soil lots which had been aggregated with Krilium lost their larger aggregates at the surface whereas the macro-aggregates formed by the other products were fairly to almost completely resistant. The numbers of *Azotobacter chroococcum* colonies obtained from well replicated platings are shown in Table 2, along with the corresponding percentages of water-stable aggregates determined at this time by the technique described by HELY and BONNIER (1953).

TABLE 2.

| Treatment                       | Per-centage active principle | Number <i>Azotobacter</i> colonies per g of soil | Percentages of aggregates |            |            |
|---------------------------------|------------------------------|--------------------------------------------------|---------------------------|------------|------------|
|                                 |                              |                                                  | 0.5-1.0 mm                | 1.0-3.0 mm | 0.5-3.0 mm |
| Control                         | —                            | 240.000                                          | 3.0                       | 2.6        | 5.6        |
| Aglusol                         | 0.05                         | 976.000                                          | 9.4                       | 44.7       | 54.1       |
|                                 | 0.10                         | 688.000                                          | 11.4                      | 59.2       | 70.6       |
| NH <sub>4</sub> OH-free Aglusol | 0.05                         | 1.616.000                                        | 9.6                       | 42.2       | 51.8       |
|                                 | 0.10                         | 1.360.000                                        | 11.3                      | 69.9       | 81.2       |
| S.R.S. 88/52                    | 0.05                         | 1.536.000                                        | 12.0                      | 52.4       | 64.4       |
|                                 | 0.10                         | 896.000                                          | 10.5                      | 62.5       | 73.0       |
| Krilium                         | 0.05                         | 928.000                                          | 11.7                      | 13.4       | 25.1       |
|                                 | 0.10                         | 1.344.000                                        | 19.9                      | 40.3       | 60.2       |

As in all other experiments of this nature the high percentages of macro-aggregation were associated with smaller numbers of *Azotobacter* colonies in the case of the first three products; this resulted presumably from the greater difficulty involved in mixing such soil lots with water for satisfactory dilution but possibly was due also in part to there being actually less nutrient available for this species in the highly aggregated soils. Here, as normally the case with this soil, 0.1% of Krilium had produced only about half as much measurable aggregation as the other three products. On this basis it is understandable why the number of colonies obtained from the soil treated with 0.1% of Krilium was very close to the numbers yielded by the second and third products at the 0.05% level. Although the dense growth of lucerne seedlings in the pots and microbiological activity over the period of a month must have resulted in much of the associated available nitrogen having been taken up, it seemed that there was still enough of this nutrient present in the soil lots treated with normal Aglusol to cause an appreciable reduction in the number of colonies obtained from this series.

Also when *Streptomyces* was studied the differences in levels of soil aggregation caused difficulties in the making of comparable dilutions of soil samples in water. All attempts made to counter this effect by finely grinding the samples in an agate mortar and mixing the soil and water vigorously in an electrically operated blender were unsuccessful. Soaking these soils for more than six hours nearly always resulted in smaller numbers of colonies appearing on the plates and soaking without agitation for one day always caused significant reduction, usually to about one-tenth of the number obtained by plating after four hours of soaking. It was noticeable that a greater proportional reduction occurred in the case of the well aggregated soils, as though the species of *Streptomyces* found in the poorly aggregated and rather compacted soil lots were less strongly aerobic than the bulk of the species in the rather loose and well aerated former soils. From all the investigations made on the effect of time of soaking on the number of colonies appearing on the plates it was decided that soaking without shaking for four hours at about 27° C. gave best results with *Streptomyces*.

Table 3 shows the type of results obtained by soaking the soil thus for four hours before dilution and plating six replicates when random samples of surface soil were taken from a replicated pot experiment

TABLE 3.

| Treatment                       | Percentage active principle | Number of <i>Streptomyces</i> colonies per g of soil | Percentage of aggregates |            |            |
|---------------------------------|-----------------------------|------------------------------------------------------|--------------------------|------------|------------|
|                                 |                             |                                                      | 0.5-1.0 mm               | 1.0-3.0 mm | 0.5-3.0 mm |
| Control                         | —                           | 304.000                                              | 3.6                      | 3.3        | 6.9        |
| Aglusol                         | 0.05                        | 331.000                                              | 9.4                      | 47.2       | 56.6       |
|                                 | 0.10                        | 155.000                                              | 8.1                      | 67.8       | 75.9       |
| NH <sub>4</sub> OH-free Aglusol | 0.05                        | 891.000                                              | 8.6                      | 48.9       | 57.5       |
|                                 | 0.10                        | 293.000                                              | 7.7                      | 76.5       | 84.2       |
| S.R.S. 88/52                    | 0.05                        | 299.000                                              | 10.2                     | 52.7       | 62.9       |
|                                 | 0.10                        | 107.000                                              | 8.3                      | 71.3       | 79.6       |
| Krilium                         | 0.05                        | 368.000                                              | 11.1                     | 14.7       | 25.8       |
|                                 | 0.10                        | 117.000                                              | 17.9                     | 38.6       | 56.5       |

where dense stands of lucerne had been growing for six weeks. The difficulty involved in comparing thus the more aggregated with the less aggregated soil lots is very obvious; allowing for this, there is no doubt that in this case the induced aggregation of the soil with its many effects had resulted in an increased number of *Streptomyces* colonies appearing on the plates. The Aglusol from which the NH<sub>4</sub>OH had been extracted was particularly favourable in this respect; the Krilium treatment gave about as many colonies as the normal Aglusol and S.R.S. 88/52 treatments but, since the Krilium-treated soils dispersed comparatively quite well in water, they almost certainly contained fewer *Streptomyces* units. Probably a greater margin of difference existed between the Krilium treated samples and those aggregated by the other products used than indicated by these figures but it was masked by the greater number of macro-aggregates actually contained in the latter. The numbers of *Streptomyces* colonies obtained from these soils were never great and the variety of colony types observed on the plates was somewhat limited.

A similar situation is shown in Table 4 which gives the results obtained from thoroughly replicated platings aimed at investigating

TABLE 4.

| Treatment                       | Percentage active principle | Number of <i>Streptomyces</i> colonies per g of soil | Percentage of aggregates |            |            |
|---------------------------------|-----------------------------|------------------------------------------------------|--------------------------|------------|------------|
|                                 |                             |                                                      | 0.5-1.0 mm               | 1.0-3.0 mm | 0.5-3.0 mm |
| Control                         | —                           | 129.000                                              | 6.5                      | 2.5        | 9.0        |
| Aggregated control              | 0.01                        | 124.000                                              | 7.9                      | 8.6        | 16.5       |
|                                 | 0.05                        | 87.000                                               | 12.4                     | 27.2       | 39.6       |
|                                 | 0.10                        | 76.000                                               | 11.2                     | 45.6       | 56.8       |
| Aglusol                         | 0.01                        | 129.000                                              | 7.2                      | 4.9        | 12.1       |
|                                 | 0.05                        | 118.000                                              | 12.0                     | 25.4       | 37.4       |
|                                 | 0.10                        | 51.000                                               | 10.4                     | 62.7       | 73.1       |
| NH <sub>4</sub> OH-free Aglusol | 0.01                        | 167.000                                              | 4.4                      | 2.8        | 7.2        |
|                                 | 0.05                        | 136.000                                              | 11.4                     | 24.0       | 35.4       |
|                                 | 0.10                        | 199.000                                              | 14.8                     | 41.1       | 55.9       |
| S.R.S. 88/52                    | 0.01                        | 157.000                                              | 10.8                     | 8.5        | 19.3       |
|                                 | 0.05                        | 159.000                                              | 13.3                     | 31.7       | 45.0       |
|                                 | 0.10                        | 195.000                                              | 12.9                     | 43.4       | 56.3       |
| Kriliun                         | 0.01                        | 229.000                                              | 2.3                      | 1.1        | 3.4        |
|                                 | 0.05                        | 188.000                                              | 7.5                      | 6.4        | 13.9       |
|                                 | 0.10                        | 176.000                                              | 10.6                     | 15.6       | 26.2       |

the *Streptomyces* situation in the surface soils of a replicated large pot experiment which had been in progress and growing a much thinner stand of lucerne for three months. In this case a mixture of the soil from the control pots was aggregated to the three normal levels by treatment with S.R.S. 88/52 so that some correction could be made for the soil suspension problem associated with aggregation treatments. The aggregation treatments by the second, third and fourth products had fairly obviously resulted in greater numbers of *Streptomyces* colonies being obtained at this stage. The pH of these soil lots three months after treatment was investigated in case an explanation might be found there for the smaller numbers of *Streptomyces* colonies obtained from those treated with normal Aglusol; the 0.01% treatment of the latter showed a pH of 6.9,



the 0.05% a pH of 7.3, the 0.10% a pH of 7.6 and the control a pH of 6.8. It was not clear whether the nitrogen added with this product had resulted in a more rapid breakdown of the synthetic resin but it did seem that at the 0.1% concentration there had been sufficient stimulation of microbial activity to give a markedly increased degree of water-stable aggregation at this time. Therefore the low numbers of colonies obtained from the soil lots treated with 0.1% of normal Aglusol could be explained, at least in part, by the greater aggregation of such soils and the associated difficulty of obtaining an appropriate suspension of their subsamples for plating.

During the course of these investigations with both *Azotobacter* and *Streptomyces* no evidence was found, nor were there even strong indications, that any of these products were inherently toxic or had given rise to toxic decomposition products in these soil samples during the first three months after their application. Furthermore, when these products other than normal Aglusol were put directly into the cooled molten media just prior to plating, no effect on the *Azotobacter* or *Streptomyces* species was apparent up to the 0.1% level; this, of course, is about the upper limit at which these substances could be used economically in commercial practice.

### S u m m a r y.

1. The effect of soil aggregation produced by synthetic soil conditioners on the occurrence of *Azotobacter* and *Streptomyces* in a highly fertile Belgian loess soil was investigated by various methods.
2. Wide differences in levels of soil aggregation introduced technical problems which made impossible the obtaining of strictly comparable results.
3. In general increased soil aggregation tended to favour an augmentation of *Azotobacter* and *Streptomyces* in the soil used and under the environmental conditions prevailing.
4. However, it seemed clear that in such investigations straight-line relationships cannot be obtained reliably because other soil micro-organisms also are favoured by such treatments and competitive effects may be emphasised from time to time.
5. No evidence was found of any inherent toxicity in the soil aggregating products used when they were employed within commercially practical limits.

---

References.

- BONNIER, CH. et HELY, F. W. 1953. Bull. Inst. Agr. et Stat. Rech. de Gembloux **21**, 14.
- BRAVO, R. A. 1952. Acta Agron. **11**, 73.
- HANOTIAUX, G. et MANIL, G. 1952. Bull. Inst. Agr. et Stat. Rech. de Gembloux **20**, 66.
- HELY, F. W. and BONNIER, CH. 1953. Antonie van Leeuwenhoek **19**, 211.
- HUSSAIN ALEEM, M. I. 1953. Plant and Soil **4**, 248.
- MANIL, G. 1952. Ann. Soc. Geol. de Belg. **75**, 153.
- MILES, A. A. and MISRA, S. S. 1938. J. Hyg. **38**, 732.
- POMALES-LEBRÓN, A. and FERNÁNDEZ, C. 1952. J. of Bact. **64**, 837.
- WANG, T. L. 1951. Plant and Soil **3**, 41.
-

(Istituto di Microbiologia Agraria e Tecnica, Università di Perugia;  
Laboratorium voor Microbiologie der Landbouwhogeschool, Wageningen).

## RECHERCHES SUR LES LEVURES DE LA FERMENTATION VINAIRE EN ITALIE

(AVEC UNE NOTE SUR LES LEVURES DU MOÛT DE RAISIN  
„FRANKENTHALER” CULTIVÉ EN SERRE)

par

**AUGUSTO CAPRIOTTI**

(Reçu le 10 Mars 1954).

La vigne croit en Italie depuis les vallées Alpines jusqu'à l'extrême pointe de la Sicile. On attache la plus grande attention à la culture de ses variétés, 90 % de la production nationale, qui atteint annuellement environ 50 millions de quintaux, étant destinés à la vinification (vins de table ordinaires, de coupage, vins de table fins, liquoreux et mousseux.)

Celle-ci ne diffère pas seulement selon la variété de la vigne, le système de culture et les conditions extrêmement variées du terrain et du climat, mais encore selon la nature des agents de la fermentation qui sont très différents dans les diverses régions.

Dans cette étude sur les levures du vin, nous nous sommes proposés deux buts:

- 1° Rapporter les résultats obtenus en Italie au cours de nombreuses recherches;
- 2° Présenter les résultats d'une étude que nous avons poursuivie aux Pays-Bas sur un moût en fermentation obtenu à partir du raisin de table „Frankenthaler”, cultivé en serre.

Depuis longtemps on sait que, dans les moûts en fermentation, on trouve tout d'abord des cellules de levures de forme apiculée, puis, après quelques jours, des cellules de forme globeuse, ovale, elliptique, qui deviendront ensuite dominantes et achèveront la fermentation. On n'a, toutefois pas encore réussi à donner une explication satisfaisante de ce phénomène. En effet, la conviction

générale est que la fermentation vinaire est induite par la „*Kloeckera apiculata*” et achevée par „*Saccharomyces ellipsoideus*”.

C'est surtout à MÜLLER-THURGAU (31) que revient le mérite d'avoir mis en évidence les différences biochimiques du processus fermentatif des deux espèces.

Tandis que la levure apiculée ne fournit, à côté de produits secondaires en quantité assez notables, des quantités modestes d'alcool, la levure elliptique fournira, au contraire des quantités élevées d'alcool et des produits secondaires en quantité souvent très restreinte. Comme les recherches de MÜLLER-THURGAU démontrèrent aussi que ces produits du métabolisme de la levure apiculée peuvent nuire au processus fermentatif de la levure elliptique, c'est à l'auteur suisse que l'oenologie est redevable de la notion de fermentations pures conduites au moyen de levures elliptiques. On a commencé à employer des levures pures, sélectionnées, et de bons résultats sont rapportés par beaucoup d'auteurs.

Les résultats de MÜLLER-THURGAU ont été confirmés et amplifiés par les recherches de MARTINAND (28) en France, et de DE' ROSSI (16) en Italie. Le premier observa dans les moûts de la Moselle, outre les levures à forme apiculée et elliptique, d'autres types de cellules de petites dimensions, de forme ronde, réunies en de petits groupes qu'il désigna du nom générique de „*Torula*”. DE' ROSSI (16), dans une série très nombreuse de moûts de l'Ombrie, observa une remarquable différence portant non seulement sur les dimensions des cellules mais surtout sur le pouvoir fermentatif. Ainsi, par exemple, une espèce de levure qu'il nomma „*Kloeckera Magna*”, pouvait fournir à peu près 10 vol. % d'alcool. Ces auteurs ont toutefois soutenu que l'on ne devait pas s'attendre à ce que la qualité des vins obtenus par fermentation pure soit toujours supérieure à celle des vins produits dans des conditions naturelles.

Plusieurs auteurs, tels que HUGUES (23), FORTI (21), MENSIO (30), MATHIEU (29), ont soutenu que l'emploi des cultures pures ne peut être généralisé, tandis que d'autres, WORTMANN (42), FABRE (19), PANTANELLI (32), CARUSO et GASPERINI (7), RICCIARDELLI (35) ont rapporté de bons résultats lorsque la fermentation pure était opérée avec des cultures sélectionnées isolées des moûts des mêmes localités où elles devaient être employées.

L'analyse des liquides fermentés a donc montré que de grandes différences existent dans le chimisme fermentatif, non seulement selon les espèces mais aussi les différentes souches d'une même espèce

et que cette diversité ne se limite pas à la production d'alcool, mais se marque aussi dans la genèse des produits secondaires, tels que les acides volatils, les alcools supérieurs et les éthers. L'influence du pourcent de sucre élevé et celle de la température ont été étudiées.

Mais toutes ces recherches sont peu cohérentes, se rapportant tantôt à la biologie des levures, tantôt à la technique oenologique.

C'est à l'Institut de Microbiologie Agricole et Technique de l'Université de Pérouse qu'appartient le mérite d'avoir commencé, il y a plus de 20 ans, des recherches systématiques sur ce sujet et de les avoir poursuivies avec les mêmes modalités techniques tant en Italie qu'à l'étranger.

Bien récemment PEYNAUD (33) a écrit: „En France, où pourtant on peut dire que l'étude des levures a été commencée, où nous avons eu des spécialistes éminents comme GUILLIERMOND, les levures du vin n'ont jamais été étudiées, spécialement dans leur ensemble. Nous n'avons rien à opposer aux nombreux travaux italiens de l'Ecole de CASTELLI de Perouse, où de l'Ecole de VERONA de Pise. Le problème a été seulement abordé par JEAN RENAUD (34), un élève de GUILLIERMOND, pour les vins du Val de la Loire. Au fond, il faut bien l'avouer, nous ne connaissons pas les levures qui font nos vins, ou nous les connaissons bien mal, et ne les connaissant pas, nous ne pouvons prétendre savoir les utiliser.”

Les recherches microbiologiques sur les moûts italiens, ont été initiées par DE'ROSSI (17) qui a étudié 82 moûts de l'Ombrie. Elles sont suivies par les recherches de CASTELLI (8) sur les moûts du Chianti classique et sur les zones limitrophes, de SANTERELLI (36) sur ceux de Colli Romani, de CASTELLI sur les moûts du Picéno (14), de BECCI sur ceux des Marches septentrionales et D'AGRIPPA (1) sur ceux des Abruzzes. Pour les moûts méridionaux c'était CASTELLI qui étudiait ceux des Pouilles (12) et ceux de la Sicile occidentale (10,13) et de Caramazza (6). CAPRIOTTI et CANTARELLI (3) ont examiné les moûts de la Province de Trévise, tandis que CAPRIOTTI a observé les moûts de la Calabre (4) et ceux du Trentino-Haut Adige (5). Les moûts de la zone typique de la production du fameux vin d'Orvieto ont été étudiés par CASTELLI (14). Il faut aussi citer les recherches que CASTELLI (15) a fait sur les moûts d'Israël et celles poursuivies dans des autres Instituts Italiens par VERONA et LUCHETTI (38), FLORENZANO (20), VERONA et FLORENZANO (39), MALAN (26, 27) et VERONA (41).



## TECHNIQUE EMPLOYÉE.

Les recherches ont toujours été faites non sur les raisins mais sur le moût, le prélevant des cuves, directement après le foulage, avant la sulfitation, afin d'obtenir des échantillons représentatifs de la constitution chimique et microbiologique. Les analyses furent faites au début de la fermentation, dans sa phase intermédiaire et dans sa phase finale, afin de connaître la succession des différentes espèces au cours du phénomène. Les analyses consistaient en l'examen microscopique du moût, immédiatement suivi par l'isolation par dissémination en employant comme moyen nutritif la gélatine au moût de raisin, préparée au moment de l'emploi en mélangeant des quantités égales de moût et de gélatine à 20 %. La gélatine au moût de raisin, en comparaison avec d'autres liquides nutritifs, donne des résultats très satisfaisants, étant très sélective et donnant des colonies très typiques, tant pour la forme et la consistance que pour la production éventuelle de pigments ou d'une liquéfaction de la gélatine. Les boîtes de Petri étaient maintenues en observation à 18-29° C. pendant 10 à 20 jours, mais normalement déjà après 5 jours on pouvait commencer les isolations, qui étaient précédées d'un examen microscopique des colonies sur les plaques au développement le plus riche; une ou plusieurs lamelles déposées sur la surface des plaques furent colorées au liqueur de Ziehl dilué, permettant la constatation d'une éventuelle différence morphologique entre les cellules des colonies. Les colonies étaient repiquées selon leur prédominance, choisissant les différents types en proportion avec leurs fréquences. Les types des colonies rarement représentés et de peu d'importance dans le procès fermentatif n'étaient pas prélevés, ou seulement à cause d'un examen purement biologique.

Dans l'ensemble des recherches conduites dans notre laboratoire jusqu'aujourd'hui, presque 500 moûts ont été analysés, résultant dans l'isolation et l'identification d'à peu près 5000 cultures pures de levures dont une bonne partie est conservée dans la Collection. La Table I présente, pour les différentes régions étudiées, le pourcentage de la fréquence noté pour les espèces de levure les plus importantes.

Les recherches ont démontré avant tout que dans la succession des diverses levures pendant la fermentation on trouve soit des espèces bien connues, soit d'autres, telles que *Saccharomyces*

TABLE I.

Pourcentage des espèces de levure trouvées dans des moûts d'origine diverse.

| Espèces                       | Italie septentr. |           | Italie centr. | Italie méridionale |       |      |               |         |       |        |      | Israël |
|-------------------------------|------------------|-----------|---------------|--------------------|-------|------|---------------|---------|-------|--------|------|--------|
|                               | ven-tri-den-tina | tré-vi-se | ⊙             | Pouilles           |       |      |               | Calabre |       | Sicile |      |        |
|                               |                  |           |               | Sept.              | Cent. | Mér. | Loco-ro-tondo | Col     | Plain | Occ.   | Mér. |        |
| <i>Kloeckera apiculata</i>    | 100              | 94,7      | 93,6          | 100                | 100   | 66,6 | 10            | 100     | 23,6  | 92     | 64,5 | 60     |
| <i>Kloeckera magna</i>        | 2,5              | —         | 19,3          | 50                 | 25    | 11,1 | —             | —       | —     | —      | 19,3 | —      |
| <i>Kloeckera africana</i>     | —                | —         | 12,9          | 25                 | 25    | 11,1 | —             | —       | —     | —      | 12,9 | —      |
| <i>Torulaspora bacillaris</i> | 12,5             | 10,5      | —             | —                  | —     | —    | —             | —       | 23,6  | —      | —    | —      |
| <i>Hans. Guilliermondii</i>   | —                | —         | —             | —                  | 33,3  | 45,9 | —             | —       | 100   | —      | 100  | 100    |
| <i>Hans. apuliensis</i>       | —                | —         | —             | —                  | 22,2  | 54,5 | —             | —       | —     | —      | —    | —      |
| <i>Sacch. ellipsoideus</i>    | 92,5             | 100       | 80,6          | 100                | 100   | 100  | 100           | 100     | 94,1  | 100    | 100  | 100    |
| <i>Sacch. Bajanus</i>         | 5                | 5,3       | 9,8           | 25                 | 25    | 55   | —             | 44      | 41,3  | 33     | 29,8 | —      |
| <i>Sacch. Mangini</i>         | —                | 21        | 0,16          | —                  | —     | —    | 20            | 33,3    | 47,2  | 41     | 23,7 | 70     |
| <i>Sacch. globosus</i>        | —                | 10,5      | —             | —                  | —     | —    | —             | 11,1    | 17,7  | 85     | —    | —      |
| <i>Sacch. italicus</i>        | 2,5              | 5,3       | 1,2           | 25                 | —     | 33,3 | 10            | 55,5    | 35,4  | 25     | —    | —      |
| <i>Sacch. Chevalieri</i>      | 2,5              | 5,3       | —             | —                  | —     | —    | 10            | 22,2    | 41,3  | 17     | —    | —      |
| <i>Sacch. oviformis</i>       | 10               | 5,3       | 5             | —                  | —     | —    | 30            | 11,1    | 17,7  | 8,5    | 40   | 40     |
| <i>Sacch. pastorianus</i>     | 65               | —         | —             | —                  | —     | —    | —             | 11,1    | 5,9   | 8,5    | —    | —      |
| <i>Torulaspora Rosei</i>      | 17,5             | 15,7      | 16,9          | 12,5               | 25    | 11,1 | 40            | 22,2    | 9,25  | 33     | —    | —      |
| <i>Torulaspora fermentati</i> | —                | —         | —             | —                  | —     | 11,1 | —             | 11,1    | —     | 17     | —    | —      |
| <i>Saccharomycodes</i>        |                  |           |               |                    |       |      |               |         |       |        |      |        |
| <i>Ludwigii</i>               | —                | 10,5      | 2,5           | —                  | —     | —    | —             | —       | —     | —      | —    | —      |

⊙ Moyennes des régions: Ombrie, Toscane (zone du Chianti), Latium, Marches, Abruzzes.

*italicus*, *Saccharomycodes bisporus*, *Zygosaccharomyces florentinus* et *Hanseniaspora apuliensis*, toutes décrites pour la première fois et dont la répartition est en rapport avec le climat des zones de production des moûts (Table I). Dans la phase initiale de la fermentation tous les chercheurs en effet, ont trouvé exclusivement des levures du type apiculé, mais, tandis que dans les moûts des zones à climat relativement froid (Italie Septentrionale et Centrale) se trouvent toujours des espèces du genre *Kloeckera* (incapable de former des spores), dans les moûts des localités à climat chaud, au contraire, on trouve des apiculés sporogènes qui appartiennent au genre *Hanseniaspora*. Ce fait important a été mis en évidence dans les premières recherches sur les moûts siciliens. En outre on a trouvé que la zone de répartition du genre *Hanseniaspora* est limitée au Nord par la partie septentrionale de la région des Pouilles, ces levures y faisant défaut.

Il est évident que les dites levures apiculées sporogènes sont strictement limitées par le climat et qu'elles préfèrent les zones à

climat chaud et très chaud. En effet dans la région des Pouilles elles augmentent en fréquence depuis la zone centrale jusqu'à la zone méridionale, toutefois sauf à Locorondo, une localité qui, bien que située dans la partie méridionale des Pouilles, se trouve à plus de 400 mètres au dessus du niveau de la mer et à un climat tempéré.

L'influence du climat est clairement mis en évidence par l'examen des moûts de Calabre: là les levures apiculées sporogènes ont été constamment trouvées avec des fréquences très élevées dans les moûts des localités littorales très chaudes, tandis qu'elles n'ont jamais été trouvées dans les moûts des localités, plus fraîches, à l'intérieur du pays.

En Sicile on trouve des levures apiculées sporogènes et non-sporogènes et en Israel, où le climat est le plus chaud, la domination du genre *Hanseniaspora* est absolue.

À côté des levures de forme apiculée on observe, au cours de la fermentation, avant tout le *Sacch. ellipsoideus* qui paraît dans presque tous les moûts de toute région. Passant des zones plus froides de l'Italie du Nord à celles de l'Italie du Sud on observe une augmentation en fréquence des espèces: *Sacch. Bajanus*, *Sacch. globosus*, *Sacch. italicus*, *Sacch. Chevalieri*. *Sacch. Mangini* est trouvé avec une fréquence très élevée dans les moûts d'Israel, bien moins en Calabre et en Sicile et avec une fréquence bien restreinte à Locorotondo. On ne le trouve que bien rarement dans l'Italie Septentrionale (zone de Treviso et Piémont), tandis qu'au Trentin, région à climat froid, il est complètement absent.

Nous tenons à mettre en relief le fait qu'aussi bien dans les moûts de la région du Trentin que dans ceux d'Israël, bien que différant fondamentalement en climat, il y a des espèces qui rivalisent en fréquence avec *Sacch. ellipsoideus*. En effet CASTELLI en Israel a trouvé *Sacch. Mangini* avec une fréquence de 70 %, tandis que dans le Trentin CAPRIOTTI a trouvé *Sacch. pastorianus* avec une fréquence de 65 %.

Pour ce qui concerne *Torulaspora Rosei* qui récemment, grâce aux travaux de CASTELLI, s'est montré d'une grande importance pour la fermentation des moûts avec une formation d'acidité volatile particulièrement réduite, il est intéressant de noter que cette espèce a été trouvée avec une fréquence variable dans presque toutes les régions d'Italie.

La Table II indique le pouvoir fermentatif maximum et minimum

TABLE II.  
Pouvoir fermentatif des espèces de levure.

| Espèces                      | Pouvoir fermentatif en degrés d'alcool |                 |
|------------------------------|----------------------------------------|-----------------|
|                              | Maximum                                | Minimum         |
| <i>Sacch. ellipsoideus</i>   | 20,5 Sicile                            | 8,5 Trevigiano  |
| <i>Sacch. pastorianus</i>    | 15,58 Calabre                          | 4,82 Trentin    |
| <i>Sacch. italicus</i>       | 15,75 Toscane                          | 11,82 Trentin   |
| <i>Sacch. Chevalieri</i>     | 15,25 Ombrie                           | 7,45 Trevigiano |
| <i>Sacch. Bajanus</i>        | 18,56 Sicile                           | 10,97 Trentin   |
| <i>Torulasporea Rosei</i>    | 13,25 Toscane                          | 5,05 Trentin    |
| <i>Kloeckera apiculata</i>   | 5,20 Sicile                            | 1,39 Trevigiano |
| <i>Torulopsis bacillaris</i> | 10,18 Marches                          | 6,73 Trevigiano |

des plus importantes espèces de levures observées dans des différentes régions d'Italie.

De l'examen de ce tableau on peut déduire l'influence exercée par le milieu sur le pouvoir fermentatif. En effet, pour les espèces indiquées, les souches qui donnent le pourcentage le plus élevé d'alcool se trouvent dans l'Italie centrale (Toscane, Ombrie et Marches) et dans l'Italie méridionale (Sicile et Calabre) où la teneur en sucre des moûts est la plus élevée. Les souches qui produisent la plus modeste quantité d'alcool se trouvent au contraire dans les moûts de l'Italie septentrionale (Trevise et Vénétie Tridentine) où le degré de sucre des moûts est plutôt bas.

Après cet exposé il paraît intéressant d'analyser les moûts obtenus de raisins cultivés dans des conditions de milieu bien différentes de celles de l'Italie. Pour cette raison nous avons examiné en Novembre 1952 au Laboratorium voor Microbiologie der Landbouwhogeschool, Wageningen, Pays Bas, un moût obtenu du raisin de table „Frankenthaler” cultivé en serre.

350 cc du moût fraîchement obtenu des raisins écrasés furent introduits stérilement dans un Erlemeyer de 500 cc, et analysés tout de suite. Le moût étant mis à fermenter à 16-18° C., les analyses furent répétées tous les deux ou trois jours, jusqu'à la fin de la fermentation qui prenait un cours plutôt lent mais régulier. Les plaques de gélatine au moût de raisin étaient incubées à 16-18° C. pendant 10 ou 15 jours. Cinq ou six jours après la préparation des plaques à dissémination on commença les isolements et 22 cultures

pures de levures furent obtenues, l'identification desquelles a été faite à l'Institut de Microbiologie Agricole de l'Université de Perugia, Italia.

Les déterminations morphologiques étaient exécutées sur des cultures en moût de raisin de 48 heures à 28° C. et sur gélose de malt de 2 ou 3 jours à 28° C., tandis que les caractères de développement furent observés dans les moûts de raisin après 30 jours à 16-18° C. et sur gélose de malt après 2 mois à 16-18° C. Le pouvoir de liquifier la gélatine fut observé dans des cultures repiquées sur gélatine au moût de raisin après 30 jours à 16-18° C. et la sporulation sur la gélose de malt et sur bloc de plâtre selon la méthode de HANSEN. Pour l'assimilation des nitrates et le développement en présence de l'alcool ethylique comme seule source de carbone la

TABLE III.

Analyses d'un moût de raisins produits en serre Néerlandaise.

| Analyses | Date des analyses | Numération des souches isolées | Souches isolées                         | Pouvoir fermentatif |
|----------|-------------------|--------------------------------|-----------------------------------------|---------------------|
| I        | 10-11-52          | 1                              | <i>Kloeckera apiculata</i>              | 5,70                |
|          |                   | 2                              | <i>Kloeckera apiculata</i>              | 5,75                |
|          |                   | 3                              | <i>Kloeckera apiculata</i>              | 5,27                |
| II       | 12-11-52          | 4                              | <i>Kloeckera apiculata</i>              | 5,65                |
|          |                   | 5                              | <i>Kloeckera apiculata</i>              | 5,97                |
|          |                   | 6                              | <i>Pichia spec.</i>                     | 2,47                |
| III      | 14-11-52          | 7                              | <i>Kloeckera apiculata</i>              | 5,52                |
|          |                   | 8                              | <i>Kloeckera apiculata</i>              | 5,26                |
|          |                   | 9                              | <i>Candida</i><br><i>Guilliermondii</i> | 4,92                |
| IV       | 16-11-52          | 10                             | <i>Kloeckera apiculata</i>              | 5,26                |
|          |                   | 11                             | <i>Kloeckera apiculata</i>              | 5,25                |
|          |                   | 12                             | <i>Hansenula anomala</i>                | 5,05                |
| V        | 18-11-52          | 13                             | <i>Hansenula anomala</i>                | 3,57                |
|          |                   | 14                             | <i>Hansenula anomala</i>                | 3,87                |
|          |                   | 15                             | <i>Sacch. ellipsoideus</i>              | 11,55               |
| VI       | 21-11-52          | 16                             | <i>Hansenula anomala</i>                | 4,37                |
|          |                   | 17                             | <i>Sacch. ellipsoideus</i>              | 12,15               |
|          |                   | 18                             | <i>Sacch. ellipsoideus</i>              | 10,62               |
| VII      | 24-11-52          | 19                             | <i>Sacch. ellipsoideus</i>              | 12,77               |
|          |                   | 20                             | <i>Sacch. ellipsoideus</i>              | 11,80               |
|          |                   | 21                             | <i>Hansenula anomala</i>                | 4,02                |
|          |                   | 22                             | <i>Sacch. ellipsoideus</i>              | 12,60               |



méthode indiquée par STELLING-DEKKER (37) a été suivie. La capacité de fermentation des sucres a été essayée sur bouillon de viande avec 2 % de sucre; le pouvoir fermentatif a été déterminé par pesage du CO<sub>2</sub> dégagé par 50 cc de moût à 28 % de sucre en Erlemeyer de 100 cc qui avait été stérilisé pendant 40 minutes à 100° C. et dont la matière coagulée et précipitée n'avait pas été éliminée. Après avoir été inoculés avec deux prises d'anse d'une culture jeune sur gélose de malt et fermés avec un bouchon de caoutchouc avec soupape Müller à l'acide sulfurique, les Erlemeyers étaient mis à fermenter à 18° C. et la diminution du poids était déterminée tous les cinq jours jusqu'à la stabilisation. La diagnose systématique a été conduite en suivant les monographies de GUILLIERMOND (22), STELLING-DEKKER (37), LODDER (24), DIDDENS et LODDER (18) et LODDER et KREGER (25). On a noté dans la table III les espèces isolées et leurs pouvoirs fermentatifs.

Nous pouvons conclure que dans la phase initiale de la fermentation du moût, obtenu de raisins produits en serre hollandaise, on trouve exclusivement *Kloeckera apiculata* à laquelle, quelques jours après, s'associent *Candida Guilliermondii* et *Pichia* species. *Hansenula anomala* se développe ensuite pendant la phase intermédiaire, tandis que plus tard, *Sacch. ellipsoideus* viendra par son association mener la fermentation à bonne fin.

En ce qui concerne *Pichia* spec. nous remarquons que cette souche n'a pas pu être rapportée aux espèces déjà décrites et nous nous proposons d'en présenter une description détaillée au plutôt. Quant à la présence de *Hansenula anomala* nous ajoutons que cette espèce dans les moûts italiens est plutôt rare. Cette espèce a été trouvée dans quelques moûts de la Toscane par CASTELLI et par FLORENZANO, tandis que dans la Vénétie Tridentine et dans quelques moûts de la Calabre elle a été trouvée par CAPRIOTTI. Plus rare encore dans les moûts italiens est la présence de *Candida Guilliermondii* et des espèces appartenant au genre *Pichia*. Quant à *Kloeckera apiculata* et *Sacch. ellipsoideus*, nous nous référons aux pages précédentes.

Quant au pouvoir fermentatif nous ajoutons toutefois que les souches de *Kloeckera apiculata* et de *Sacch. ellipsoideus*, isolées du moût hollandais, ont produit des quantités d'alcool voisines de celles que les mêmes espèces présentent dans nos moûts de l'Italie centrale.

## Résumé.

On a étudié la répartition géographique des différentes espèces de levures intervenant dans la fermentation des vins italiens.

L'influence du climat a été démontrée: des espèces fréquentes dans certaines régions sont absentes dans d'autres.

On a comparé, à celui des moûts oenologiques, l'assortiment des levures présentes dans un moût de raisin de table néerlandais, cultivé en serre.

## Bibliographie.

1. AGRIPPA, M. 1949. Tesi di laurea. Facoltà di Agraria dell'Università di Perugia.
2. BECCI, M. 1949. Tesi di laurea. Facoltà di Agraria dell'Università di Perugia.
3. CAPRIOTTI, A. e CANTARELLI, C. 1952. Riv. Vit. e di Enologia **5**, 213, 253, 288.
4. CAPRIOTTI, A. 1952. Ann. Facoltà d'Agraria di Perugia **8**, 64.
5. CAPRIOTTI, A. 1953. Atti Acc. It. Vite e Vino, Siena **5**, 262.
6. CARAMAZZA, I. 1947. Ann. Facoltà d'Agraria di Perugia **4**, 170.
7. CARUSO, G. e GASPARINI, G. 1948. Atti Reale Acc. dei Georgofilli, Firenze **76**, 213.
8. CASTELLI, T. 1939. Nuovi Annali dell'Agr. Italiana **19**, 47.
9. CASTELLI, T. 1939. Nuovi Annali dell'Agr. Italiana **19**, 85.
10. CASTELLI, T. 1947. Ann. Facoltà d'Agraria di Perugia **4**, 45.
11. CASTELLI, T. 1947. L'Italia Agricola R.E.D.A., Roma **84**, 521.
12. CASTELLI, T. 1948. Suppl. de la Rivista Scientifica **18**, 67.
13. CASTELLI, T. 1950. Riv. Vit. e di Enologia **3**, 181, 217.
14. CASTELLI, T. 1949. Riv. Vit. e di Enologia **2**, 223.
15. CASTELLI, T. 1951. Bull. Research Council of Israel **1**, 54.
16. DE'ROSSI, G. 1920. Stazioni Sperimentali Agrarie Italiane **53**, 233.
17. DE'ROSSI, G. 1935. Rel. IV° Congr. Int. Vite e Vino, Lausanne p. 1.
18. DIDDENS, M. und LODDER, J. 1940. Die anaskosporogenen Hefen II, Amsterdam.
19. FABRE, H. 1894. C. R. d l'Acad. d. Sc. voir: CASTELLI, T. 1951. Rel. VI° Cong. Enotec. Naz. Milano. p. 15.
20. FLORENZANO, G. 1949. Ann. Sper. Agraria **3**, 887.
21. FORTI, C. 1901. Ann. Soc. Chim. Milano **7**.
22. GUILLIERMOND, A. 1912. Les levures. Paris.
23. HUGUES, C. 1894. Stazioni Sperimentali Agrarie Italiane **27**, 7.
24. LODDER, J. 1934. Die anaskosporogenen Hefen I. Amsterdam.
25. LODDER, J. and KREGER-VAN RIJ, N. J. W. 1952. The Yeasts. Amsterdam.
26. MALAN, C. 1949. Atti Acc. It. Vite e Vino, Siena **3**, 338.
27. MALAN, C. 1953. Atti Acc. It. Vite e Vino, Siena **5**, 345.

28. MARTINAND, V. 1909. *Rev. de vit.* **32**, 174.
  29. MATHIEU, L. 1911. *Rev. de vit.* **35**, 342; **36**, 119.
  30. MENSIO, C. 1924. *Atti Cons. Naz. Chim. Industriale* **4**.
  31. MÜLLER-THURGAU, H. 1894-1897. *Jahresber. der Schweiz. Versuchsanstalt in Wädenswil* **5-7**. Voir: DE'ROSSI, G. 1935. *Ref.* **17**.
  32. PANTANELLI, N. 1906. *Stazioni Sperimentali Agrarie Italiane* **39**, 543.
  33. PEYNAUD, E. 1952. *Revue des Ferment. et des Ind. Alimentaires* **7**, 71.
  34. RENAUD, J. 1941. *Thèse Fac. des Sciences, Paris*.
  35. RICCIARDELLI, N. 1899. *Bul. Not. Agr. Ministero Agr. Roma* **21**, 359.
  36. SANTARELLI, B. 1942. *Ann. Facoltà d'Agraria di Perugia* **1**, 161.
  37. STELLING-DEKKER, N. M. 1931. *Die sporogenen Hefen*. Amsterdam.
  38. VERONA, O. e LUCHETTI, G. 1936. *Bul. R. Ist. Sup. Agrario di Pisa* **12**, 256.
  39. VERONA, O. e FLORENZANO, G. 1947. *Ricerca Scientifica e Ricostituzione* **17**, 1401.
  40. VERONA, O. e FLORENZANO, G. 1947. *d'Agricoltura Italiana* **47**, 113.
  41. VERONA, O. 1951. *Annali Facoltà Agraria di Pisa* **12**, 523.
  42. WORTMANN, J. 1900. *Landwirtschaftliche Jahrbücher*, Berlin **29**.
-

(Aus dem Institut für landw. Bakteriologie und Gärungsbiologie, Eidg. Techn. Hochschule, Zürich).

**UNTERSUCHUNGEN ÜBER DIE PHYSIOLOGIE  
DER WEINHEFEN. V. MITTEILUNG  
ÜBER DIE KOHLENDIOXYDPRODUKTION DER  
„FENDANT“-WEINHEFE IN STICKSTOFF BZW.  
ARGON, LUFT UND STICKSTOFF- BZW.  
ARGON-LUFT-GEMISCH <sup>1)</sup>**

von

**T. WIKÉN und O. RICHARD**

(Eingegangen am 17. März 1954).

Seit der Entdeckung PASTEURS (50), dass sich gewisse Hefen bei Luftabschluss eine Zeitlang vermehren können, ist die Wechselwirkung zwischen dem Wachstum bzw. der Vermehrung der Hefen und deren Atmung bzw. Gärung (Dissimilation des Zuckers mit bzw. ohne O<sub>2</sub>-Aufnahme) in ihrer Abhängigkeit von der Konzentration bzw. Zufuhr und Verteilung des gasförmigen Sauerstoffs der Gegenstand zahlreicher Untersuchungen gewesen. Es stellte sich dabei heraus, dass die Assimilation des Zuckers in Gegenwart geeigneter Stickstoffverbindungen unter Bildung von Plasma und Zellwandsubstanz (Wachstum bzw. Vermehrung der Hefe) sowie die Speicherung desselben in Abwesenheit verwertbarer Stickstoffquellen unter Synthese von höheren Kohlehydraten wie Glykogen und Trehalose sowie Fett (Trockensubstanzzunahme ohne Erhöhung der Zellenzahl der Hefe, Hefeverfettung) durch reichliche Sauerstoffzufuhr gefördert werden (siehe EULER und LINDNER (13) S. 69 und 239 ff., LUNDIN (37, 38), MYRBÄCK und OERTENBLAD (44), BERNHAUER (2) S. 97 ff. und 104 ff., ENEBO, ANDERSON und LUNDIN (11), JÖRGENSEN und HANSEN (26) S. 18, 91 und 244, ELANDER und MYRBÄCK (10), NILSSON und ALM (47), NILSSON (45), LÜERS (36) S. 626 und 649 ff., WHITE und MUNNS (71), AMERINE

<sup>1)</sup> I, II, III, IV. Mitteilung: Antonie van Leeuwenhoek 17, 209, 1951; 18, 31, 1952; 18, 293, 1952; 19, 279, 1953.

und JOSLYN (1) S. 298, JOSLYN (27) S. 269 ff., HAEHN (20) S. 119, 267, 279 und 371ff., RIPPEL-BALDES (55) S. 32, 163, 212 und 351, FINK und ROSS (18), etc.). Bekanntlich gewinnt man heutzutage die Bäckerei- wie auch die Futter- und Fetthefe nach dem Lüftungsverfahren, wobei allerdings der günstige Einfluss der Lüftung auf die Vermehrungsgeschwindigkeit der Hefe bzw. ihre Eiweiss- und Fettsynthese nicht nur auf die reichliche Zufuhr von fein und gleichmässig verteiltem Sauerstoff und die Umsteuerung des Stoffwechsels der Hefezellen durch dieses Gas zurückzuführen ist, sondern auch dadurch bedingt ist, dass die Anreicherung von hemmenden Stoffwechselprodukten wie Aethylalkohol und Kohlendioxyd im Medium auf mechanischem Wege verhindert wird und die Oberfläche der Zellen ständig mit Nähr- und Wachstoffsstoffhaltigem Substrat in Berührung kommt (siehe BRAUN und PFUNDT (4), FINK, LECHNER und HEINISCH (17), FINK und LECHNER (16), PFUNDT (51), DE BECZE und LIEBMAN (8), OLSON und JOHNSON (48), MENZINSKY (39), etc.).

In der Weinkelterei konnte festgestellt werden, dass die Lüftung des mit Hefe geimpften Traubensafts im frühen Stadium der Gärung eine günstige Wirkung auf den Zuckerabbau ausübt. Diese beruht nach RIBÉREAU-GAYON u. Mitarb. (53, 54) sowie AMERINE und JOSLYN (1, S. 298) auf der Steigerung der Vermehrungsgeschwindigkeit der Hefe in Anwesenheit von Sauerstoff. Wenn dagegen der Traubensaft erst in späteren Stadien der Gärung mit Sauerstoff versetzt wird, lässt sich kein fördernder Einfluss auf das Wachstum der Hefezellen und den Abbau des Zuckers nachweisen.

Zahlreiche Forscher haben sich mit der Umsteuerung des Zuckerdissimilationstypus der Hefen in ihrer Abhängigkeit von der Sauerstoffspannung in Gärungs- und Atmungsversuchen mit ruhenden Zellen bzw. von der Zufuhr und Verteilung des Sauerstoffs während der Züchtung des Zellmaterials, welches in derartigen Versuchen zur Verwendung kam, beschäftigt. In gewissen Fällen gelang es dabei, gärschwache Hefen durch Züchtung bei gedrosseltem Luftzutritt in gärstarke Hefen umzuwandeln. Andererseits konnte das kräftige Gärvermögen zymasereicher Rassen durch Züchtung unter starker Lüftung praktisch ganz zum Verschwinden gebracht werden (siehe HAYDUCK und HAEHN (21), MEYERHOF (41), WARBURG (66), HAEHN (20) S. 265 ff., etc.). Eine solche Verschiebung im Verhältnis der Atmung zur Gärung je nach der Grösse der Luftzufuhr während der Vermehrung der Hefen wird von einigen



Forschern bestritten. Sie konnten zwar einen deutlichen Einfluss der Lüftungsbedingungen auf den Gasstoffwechsel der untersuchten Hefen feststellen, aber mit einer Steigerung des Gärvermögens lief immer eine Erhöhung der Atmungsintensität parallel. Eine Verminderung der Gärungsgeschwindigkeit war ferner stets mit einer Abnahme der Atmungsgeschwindigkeit verknüpft. Eine Umsteuerung oder Umstimmung des Kohlehydratstoffwechsels der Hefen in dem Sinne, dass einer von diesen Prozessen zugunsten des anderen zurücktritt, konnte nicht nachgewiesen werden. Das Verhältnis der Gärungsgeschwindigkeit zur Atmungsgeschwindigkeit stellte bei den betreffenden Hefen einen konstanten Wert dar (siehe TRAUTWEIN und WASSERMANN (62), WINDISCH (76) S. 265 ff., etc.).

Die oben erwähnte Erscheinung, dass die Gärung (Dissimilation des Zuckers ohne  $O_2$ -Aufnahme) der Hefen und zahlreicher anderer Zellarten in Gegenwart des elementaren Sauerstoffs mehr oder weniger stark unterdrückt wird und die Zellen ihren Energiebedarf in grösserem Umfang durch Atmung (Dissimilation des Zuckers mit  $O_2$ -Aufnahme) decken, wird bekanntlich als der PASTEUR- oder PASTEUR-MEYERHOF-Effekt bezeichnet. Bezüglich der umfangreichen Literatur, welche diese Reaktion von biochemischen und physiologischen Gesichtspunkten behandelt, verweisen wir auf die Arbeiten von HOOGERHEIDE (25), MEYERHOF (42), LIPMANN (35), HÖBER (23) S. 431 ff., SUMNER und SOMERS (59) S. 201 und 374 ff., WERKMAN und WILSON (70) S. 351 ff. sowie HAEHN (20) S. 265 ff.

In den letzten Jahren wurde nun nachgewiesen, dass die Gärung bei gewissen Mikroorganismen durch den elementaren Sauerstoff nicht gehemmt wird sondern vielmehr stimuliert werden kann. So wurde ein fördernder Einfluss dieses Gases von CUSTERS (7)<sup>1)</sup> auf die alkoholische Gärung bei der asporogenen Hefe *Brettanomyces clausenii*, von CANTINO (5) auf die Milchsäure-Bernsteinsäure-Gärung beim Algenpilz *Blastocladia pringsheimii* und von WAKSMAN und FOSTER (siehe FOSTER (19) S. 290 ff.) auf die Milchsäure-Gärung bei einem *Rhizopus*-Stamm (MX) festgestellt. CUSTERS, der Messungen der  $O_2$ -Afnahme und der aeroben bzw. anaeroben Entwicklung von Gärungs- $CO_2$  mit Hilfe der manometrischen Methode nach Warburg ausführte, fand nun, dass das Auftreten des „negativen PASTEUR-MEYERHOF-Effekts“ von der Vorgeschichte des Zellmaterials stark

<sup>1)</sup> Wir danken Herrn Professor Dr. A. J. KLUYVER, Delft, herzlich, dass er uns am VI. Internationalen Mikrobiologenkongress in Rom (6.—12. September 1953) auf diese Arbeit aufmerksam gemacht hat.

abhängig ist. Während beispielsweise beim Zellmaterial von 3tägigen *Brettanomyces*-Kulturen auf Kreide-Malzagar-Platten eine günstige Wirkung des Sauerstoffs eindeutig feststellbar ist, tritt beim Material aus 7 Tage alten Kulturen die PASTEUR-MEYERHOF-Reaktion auf.

Die gärstarken Hefen, z.B. die untergärige Bierhefe, sind bekanntlich wesentlich reicher an Vitamin B<sub>1</sub> als die gärschwachen Typen, z.B. die nach dem Lüftungsverfahren erzeugte *Torula*-Futterhefe. Werden die ersten unter reichlicher Luftzufuhr gezüchtet, so nimmt ihr Aneuringehalt beträchtlich ab, während bei den letzten durch Vermehrung unter gedrosseltem Luftzutritt eine erhebliche Steigerung des Aneuringehalts erzielt werden kann (siehe FINK und JUST (15)).

Es konnte ferner gezeigt werden, dass der Typus des Cytochromspektrums bei den Hefen sehr stark von den Lüftungsbedingungen, unter welchen die Züchtung stattfindet, abhängig ist (siehe FINK und BERWALD (14) sowie EPHRUSSI und SLONIMSKI (12)). Beispielsweise werden unter- und obergärige Brauereihefen, welche das zweibandige Cytochromspektrum (Gärungshefetypus) aufweisen, durch längere Fortzüchtung unter reichlichem Luftzutritt so verändert, dass sie das vierbandige Spektrum der nach dem Lüftungsverfahren erzeugten Bäckerhefe (Atmungshefetypus) annehmen. Diese Umwandlung des Spektraltypus ist aber nicht unbedingt an eine Vermehrung der Hefe gebunden, sondern kann auch in ruhenden Hefezellen („resting cells“) stattfinden (siehe CHIN (6)).

In den oben erwähnten Untersuchungen über den Einfluss des Sauerstoffs auf die Wechselwirkung zwischen Vermehrung, Atmung und Gärung bei den Hefen enthielt die Gasphase praktisch reinen Stickstoff oder Sauerstoff bzw. native oder synthetische Luft (1 Vol. O<sub>2</sub> + 4 Vol. N<sub>2</sub>). Der Gasstoffwechsel der aeroben Hefesuspensionen wurde somit durchgehend bei hohen Sauerstoffkonzentrationen untersucht. In Zusammenhang mit unseren eigenen Untersuchungen über die Physiologie der Weinhefen (72, 73, 74) schien es nun von Interesse, einerseits das Auftreten bzw. Ausbleiben des PASTEUR-MEYERHOF-Effekts bei der Rasse „Fendant“ festzustellen, andererseits die Wirkung der Luft bzw. minimaler Mengen Sauerstoff auf die Vergärung der Glucose durch ruhende Zellen dieser Hefe zu studieren. Im Hinblick auf die Ergebnisse unserer früheren Gärversuche mit Kulturweinhefen (74) wurde dabei das Alter der Kulturen, welche das Zellmaterial lieferten,

berücksichtigt und ferner die Bedeutung der Dauer der  $N_2$ -Begasung für die physiologische Aktivität der Zellen geprüft.

### METHODIK.

Die von uns zur Gewinnung des Zellmaterials der Kulturweinhefen benützten Nährlösungen, Kulturgefäße und Erntemethoden sowie die Details der in den Gärversuchen verwendeten Methodik wurden bereits ausführlich beschrieben (72, 74). Hier sei nur erwähnt, dass das „Fendant“-Material in Fernbach-Kolben in Nährlösung C ohne irgend einen Zusatz von Wuchsstoffen bei 20° C. oder 23° C. gezüchtet wurde. Diese enthält nebst Mineralsalzen und Kaliumcitrat-Puffer vitaminfreies Caseinhydrolysat als Stickstoffquelle und Glucose als Kohlenstoff- und Energiequelle. Die Geschwindigkeit der Kohlendioxydproduktion der gewaschenen Hefezellen wurde volumetrisch in der Apparatur nach VON EULER, MYRBÄCK, NILSSON und ALM (46) bei 25° C. gemessen, wobei die Schüttelgeschwindigkeit 100 volle Schwingungen (je 100 Hin- und Herbewegungen) pro Minute betrug. Als Gärgefäße dienten Erlenmeyer-Kolben von etwa 50 ml Gesamtvolumen mit einer seitlich eingeschliffenen drehbaren Ampulle. Diese Gefäße enthielten 2,0 ml Hefesuspension im Hauptraum und 1,0 ml Glucoselösung in der Seitenampulle. Dies entsprach 300 mg gewaschener frischer Hefe bzw. 200 mg wasserfreiem Zucker. Zur Herstellung der Hefesuspension bzw. Glucoselösung benutzten wir einen 0,16 M Bernsteinsäure-Natriumsuccinat-Puffer vom pH 4,9 (siehe BRANDT (3)). Der zur Begasung der Gärkolben verwendete Stickstoff wurde von Sauerstoff befreit, indem wir das Gas durch alkalische Pyrogallol-lösung (KÜSTER (34) S. 71) oder alkalische Natriumhyposulfit- (Natriumhyposulfit-) Lösung (TREADWELL (63) S. 667) und nachher über rotglühendes reduziertes Kupfer im elektrischen Ofen bei 400-450° C. strömen liessen. Das zur Begasung ebenfalls benutzte Argon wurde in der gleichen Weise behandelt.

Der durch die geschüttelten Gärgefäße strömende Stickstoff bzw. das Argon wurden auf  $O_2$ -Freiheit mit Hilfe einer alkalischen Glucose-Methylenblau-Lösung nach FILDES und McINTOSH (siehe MEYER und PIETSCHMANN (40) S. 1538 ff.) geprüft. Diese Lösung befand sich in einem an das Aus-

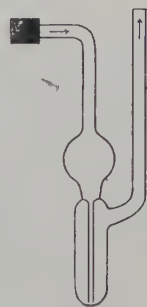


Abb. 1. Gefäß für die Prüfung der Atmosphäre der Gärkolben auf  $O_2$ -Freiheit.

laufrohr der Gasbüretten angeschlossenen Gefäß, dessen Konstruktion aus Abb. 1 ersichtlich ist. Zur Reduktion (Entfärbung) des Methylenblaus wurde der untere Teil der an die Büretten befestigten Gefäße durch schnelles Eintauchen in ein Glycerinbad erhitzt.

### ERGEBNISSE UND DISKUSSION.

Versuche mit Zellmaterial aus jungen und alten Kulturen in Stickstoff bzw. Luft und Stickstoff-Luft-Gemisch. Alkalische Pyrogallollösung als  $O_2$ -Absorptionsmittel.

Im ersten Versuch kam Hefematerial aus 20 Stunden alten Kulturen zur Verwendung. Vor dem Zusetzen der Glucoselösung wurden die Zellsuspensionen in den Gärgefäßen in ruhender Luft

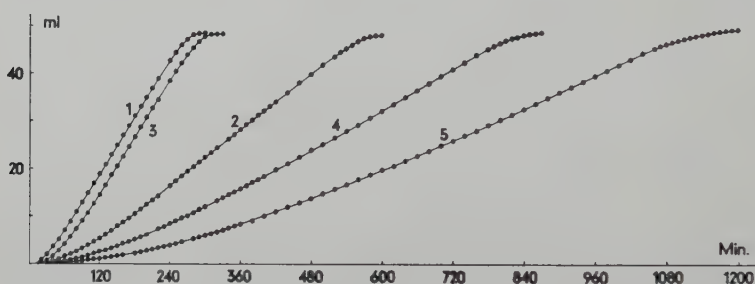


Abb. 2. Die Abhängigkeit der Vergärung der Glucose von der Dauer der  $N_2$ -Gasung und vom Zusatz einer kleinen Menge Luft unmittelbar vor dem Einkippen der Glucoselösung. Zellmaterial aus 20 Stunden alten Kulturen.

Alkalische Pyrogallollösung als  $O_2$ -Absorptionsmittel.

Behandlung der Hefesuspension vor dem Zusetzen der Zuckerlösung:

1. 40 Min. Schütteln mit ruhender Luft.
2. 40 Min. Schütteln mit strömendem  $N_2$ .
3. 40 Min. Schütteln mit strömendem  $N_2$ , Zusatz von 3 ml Luft.
4. 40 Min. Schütteln mit strömendem  $N_2$ , 20 Min. Schütteln in ruhender  $N_2$ -Atmosphäre.
5. 40 Min. Schütteln mit strömendem  $N_2$ , 60 Min. Schütteln in ruhender  $N_2$ -Atmosphäre.

1, 3 und 5: 2 Parallelen. 2 und 4: 3 Parallelen.

bzw. strömendem Stickstoff während 40 Min. geschüttelt. Einige der mit Stickstoff behandelten Suspensionen wurden vor dem Einkippen der Zuckerlösung für weitere 20 bzw. 60 Min. in ruhender Stickstoffatmosphäre geschüttelt, während in anderen Fällen 3 ml Luft, d.h. 0,6 ml Sauerstoff, zugesetzt wurden. Dies entsprach einer  $O_2$ -Konzentration in der Gasphase der betreffenden Gärkolben von ca. 1 Volumen-%. Aus Abb. 2 geht hervor, dass die Gär-  
geschwindigkeit unter streng anaeroben Bedingungen (Kurven 2,



4 und 5) viel kleiner ist als unter aeroben Bedingungen (Kurve 1), wobei sie mit zunehmender Dauer der  $N_2$ -Behandlung kräftig abnimmt. Die in Luft geschüttelte Hefesuspension entwickelt in der approximativ geradlinigen Gärphase 11,9 ml  $CO_2$ /300 mg feuchte Hefe und Stunde, während die mit Stickstoff während 40 bzw. 40 + 20 und 40 + 60 Min. behandelten Suspensionen in der gleichen Phase nur 5,9 bzw. 4,3 und 3,3 ml produzieren. Die Zeitspanne, welche zur vollständigen Vergärung des Zuckers nötig war, betrug für die aerobe Suspension 300 Min. und für die anaeroben Zellen 600 bzw. 870 und 1200 Min. Es ist ferner ersichtlich, dass die Gärungshemmung, welche die Hefesuspension nach Begasung während 40 Min. aufweist (Kurve 2), durch den Zusatz einer geringen  $O_2$ -Menge praktisch quantitativ aufgehoben wird, indem die Geschwindigkeit der  $CO_2$ -Bildung in Stickstoff-Luft-Gemisch 12,0 ml/300 mg feuchte Hefe und Stunde beträgt und zum quanti-

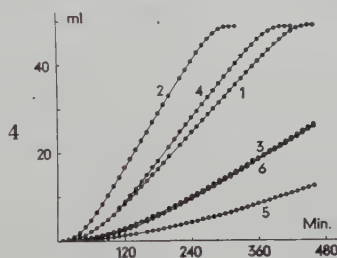
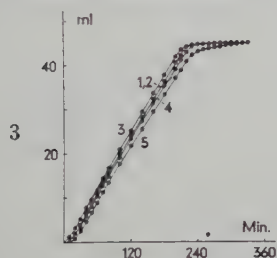


Abb. 3. Die Abhängigkeit der Vergärung der Glucose von der Dauer der  $N_2$ -Gasung und vom Zusatz einer kleinen Menge Luft unmittelbar vor dem Einkippen der Glucoselösung. Zellmaterial aus 72 Stunden alten Kulturen. Alkalische Pyrogallollösung als  $O_2$ -Absorptionsmittel.

Behandlung der Hefesuspension vor dem Zusetzen der Zuckerlösung:

1. 40 Min. Schütteln mit ruhender Luft.
2. 40 Min. Schütteln mit strömendem  $N_2$ .
3. 40 Min. Schütteln mit strömendem  $N_2$ , Zusatz von 3 ml Luft.
4. 40 Min. Schütteln mit strömendem  $N_2$ , 20 Min. Schütteln in ruhender  $N_2$ -Atmosphäre.
5. 40 Min. Schütteln mit strömendem  $N_2$ , 60 Min. Schütteln in ruhender  $N_2$ -Atmosphäre.

1, 3 und 5: 2 Parallelen. 2 und 4: 3 Parallelen.

Abb. 4. Die Abhängigkeit der Vergärung der Glucose von der Dauer der  $N_2$ -Gasung und vom Zusatz einer kleinen Menge Luft unmittelbar vor dem Einkippen der Glucoselösung. Zellmaterial aus 20 Stunden alten Kulturen. Alkalische Pyrogallollösung als  $O_2$ -Absorptionsmittel.

Behandlung der Hefesuspension vor dem Zusetzen der Zuckerlösung:

1. 40 Min. Schütteln mit strömendem  $N_2$ .
2. 40 Min. Schütteln mit strömendem  $N_2$ , Zusatz von 3 ml Luft.
3. 40 Min. Schütteln mit strömendem  $N_2$ , 20 Min. Schütteln in ruhender  $N_2$ -Atmosphäre.
4. 40 Min. Schütteln mit strömendem  $N_2$ , 20 Min. Schütteln in ruhender  $N_2$ -Atmosphäre, Zusatz von 3 ml Luft.
5. 40 Min. Schütteln mit strömendem  $N_2$ , 60 Min. Schütteln in ruhender  $N_2$ -Atmosphäre.
6. 40 Min. Schütteln mit strömendem  $N_2$ , 60 Min. Schütteln in ruhender  $N_2$ -Atmosphäre, Zusatz von 3 ml Luft.

2 Parallelen.



tativen Verbrauch der Glucose nur 320 Min. nötig sind (Kurve 3). Die Gesamtvolumina, um welche sich die gärenden Systeme beim scharfen Abbiegen der Gärkurven vergrößert hatten, waren durchschnittlich für Luft 48,4 und für Stickstoff-Luft-Gemisch 48,2 ml. Für Stickstoff betrugen sie im Durchschnitt 48,0, 48,3 und 48,5 ml.

Im zweiten Versuch wurde das Gärvermögen von Hefematerial aus 72 Stunden alten Kulturen untersucht, wobei die Hefesuspension vor dem Zusetzen des Zuckers in der gleichen Weise behandelt wurde wie im ersten Versuch. Die Ergebnisse liegen in Abb. 3 vor. Es ist ersichtlich, dass die Gärfähigkeit der aus alten Kulturen stammenden „Fendant“-Hefe von der Vorbehandlung der Zellsuspensionen praktisch unabhängig ist. Im annähernd linearen Teil der Gärkurve konnte für die in Luft geschüttelte Hefe eine Gärgeschwindigkeit von 12,4 ml/300 mg feuchte Hefe und Stunde festgestellt werden (Kurve 1), während die entsprechenden Werte für die mit Stickstoff während 40 bzw. 40 + 20 und 40 + 60 Min. behandelten Suspensionen 12,4 bzw. 11,7 und 11,6 ml betrugen (Kurven 2, 4 und 5). In Anwesenheit von ca. 1 Volumen-% Sauerstoff in der Gasphase entwickelte die in strömendem Stickstoff geschüttelte Hefe 12,5 ml CO<sub>2</sub>/300 mg feuchte Hefe und Stunde (Kurve 3). Abgesehen von der Suspension, welche für 40 + 60 Min. mit Stickstoff behandelt wurde, hatte die Hefe den Zucker bereits in 260 Min. vergoren. Für die erwähnte Suspension betrug die zum vollständigen Glucoseverbrauch benötigte Zeit 340 Min.

Die Inaktivierung des Zymasystems, die bei ruhenden Zellen aus jungen Kulturen der Kulturweinhafe „Fendant“ durch Stickstoff hervorgerufen wird, sowie die Reaktivierung dieses Enzymsystems, welche in Anwesenheit geringer Mengen Sauerstoff bei den gleichen Zellen festgestellt werden kann, lassen sich mithin bei Hefematerial aus alten Kulturen nicht oder nur in geringem Ausmasse nachweisen. In diesem Zusammenhang ist aber auch hervorzuheben, dass beim letzten Material kein PASTEUR-MEYERHOF-Effekt auftritt.

Wie bereits erwähnt, kann die nach N<sub>2</sub>-Begasung während 40 Min. bei „Fendant“-Zellen aus jungen Kulturen eintretende Gärungshemmung durch sehr kleine O<sub>2</sub>-Mengen praktisch quantitativ beseitigt werden. In einem dritten Versuch beabsichtigten wir nun abzuklären, ob dies auch bei den Hefesuspensionen, welche vor dem Einkippen der Glucoselösung für längere Zeitspannen (40 + 20 bzw. 40 + 60 Min.) mit sauerstofffreiem Stickstoff behandelt werden,

der Fall ist. Aus den Versuchsergebnissen, welche in Abb. 4 aufgeführt sind, geht hervor, dass sich die sehr starken Gärungshemmungen, welche nach den letzterwähnten  $N_2$ -Behandlungen einsetzen, nur zum Teil durch kleine  $O_2$ -Mengen aufheben lassen. Nach Schütteln während 40 bzw.  $40 + 20$  und  $40 + 60$  Min. in Stickstoff wiesen die Hefesuspensionen unter streng anaeroben Bedingungen in der approximativ geradlinigen Phase Gärungsgeschwindigkeiten von 8,1 bzw. 4,6 und 2,5 ml  $CO_2$ /300 mg feuchte Hefe und Stunde auf (Kurven 1, 3 und 5). Die entsprechenden Werte in Anwesenheit von ca. 1 Volumen-% Sauerstoff betrugen 12,4 bzw. 9,5 und 4,6 ml (Kurven 2, 4 und 6). Zum vollständigen Umsatz des Zuckers brauchten die in sauerstoffhaltigem Stickstoff gärenden Zellsuspensionen wesentlich kürzere Zeiten als die entsprechenden Suspensionen in sauerstofffreier Stickstoffatmosphäre (40 Min. Schütteln in Stickstoff: 310 bzw. 460 Min.;  $40 + 20$  Min. Schütteln in Stickstoff: 400 bzw.  $\gg 460$  Min.).

Beim Schütteln von ruhenden Hefesuspensionen, deren Zellen aus jungen „Fendant“-Kulturen stammen, in sauerstofffreiem Stickstoff finden mithin nebst der reversiblen, in Anwesenheit geringer  $O_2$ -Mengen rückgängigen Inaktivierung bestimmter Zymasekomponenten in gewissem Ausmasse Prozesse statt, die zur irreversiblen Zerstörung von Zellbestandteilen führen, welche für die Gärung (Dissimilation des Zuckers ohne  $O_2$ -Aufnahme) unerlässlich sind.

Versuche mit Zellmaterial aus jungen Kulturen in Stickstoff bzw. Luft. Alkalische Natriumhydrosulfit- (Natriumhyposulfit-) Lösung als  $O_2$ -Absorptionsmittel.

Bei der Absorption des Sauerstoffs durch eine alkalische Pyrogalllösung entstehen bekanntlich unter Umständen kleine Mengen Kohlenmonoxyd (siehe KÜSTER (34) S. 71, TREADWELL (63) S. 666, HOLLEMAN (24) S. 368, WAGNER (65) S. 36). Die Lösung, welche in unseren Versuchen zur Verwendung kam, hatte eine Zusammensetzung (1 Volumen 25 % Pyrogalllösung + 6 Volumina 60 % Kalilauge), bei welcher dieses Gas nicht oder nur in Spuren gebildet werden sollte. Die Prüfung mit Hilfe eines Fega-Teströhrchens zeigte ferner, dass der  $CO$ -Gehalt des zur Begasung der Gärkolben verwendeten Stickstoffs wesentlich kleiner war als derjenige gewöhnlicher Laboratoriumsluft. Gemäss OPPENHEIMER und STERN (49, S. 72) wird die alkoholische Gärung durch Kohlen-

monoxyd nicht beeinflusst. In Anbetracht der Tatsache aber, dass diese Verbindung eine hemmende Wirkung auf die Aktivität zahlreicher Enzyme der oxydativen und anoxydativen Dissimilationsprozesse (mit bzw. ohne  $O_2$ -Aufnahme) ausübt, wobei der Stoffwechsel der betreffenden Zellen häufig umgesteuert wird (WARBURG (67), WARBURG und NEGELEIN (68), KUBOWITZ und HAAS (33), KEMPNER (28), KEMPNER und KUBOWITZ (29), KUBOWITZ (32), HOBERMAN und RITTENBERG (22), WARING und WERKMAN (69), SIMON (57), EBERSOLE, GUTTENTAG und WILSON (9); siehe ferner OPPENHEIMER und STERN (49) S. 72 ff., PORTER (52) S. 545 und 832, SUMNER und SOMERS (59) S. 223, 231 und 329, WERKMAN und WILSON (70) S. 487, 495, 511, 526 und 527, RIPPEL-BALDES (55) S. 171, etc.), wurde in einigen Versuchen vorsichtshalber die alkalische Pyrogallollösung durch eine alkalische Lösung von Natriumhydrosulfit (Natriumhyposulfit,  $Na_2S_2O_4 \cdot 2 H_2O$ ) ersetzt (siehe TREADWELL (63) S. 667, WAGNER (65) S. 37). Die kleinen

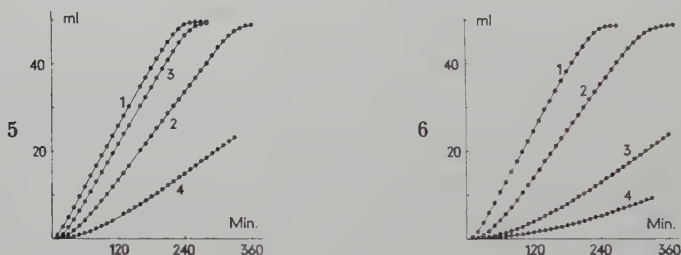


Abb. 5. Die Abhängigkeit der Vergärung der Glucose von der Dauer der  $N_2$ -Gasung vor dem Einkippen der Glucoselösung. Zellmaterial aus 18 Stunden alten Kulturen. Alkalische  $Na_2S_2O_4$ -Lösung als  $O_2$ -Absorptionsmittel.

Behandlung der Hefesuspension vor dem Zusetzen der Zuckerlösung:

1. 30 Min. Schütteln mit strömender Luft.
  2. 30 Min. Schütteln mit strömendem  $N_2$ .
  3. 30 Min. Schütteln mit strömendem  $N_2$ , 5 Min. Schütteln mit strömender Luft.
  4. 60 Min. Schütteln mit strömendem  $N_2$ .
- 2 Parallelen.

Abb. 6. Die Abhängigkeit der Vergärung der Glucose von der Dauer der  $N_2$ -Gasung vor dem Einkippen der Glucoselösung. Zellmaterial aus 18 Stunden alten Kulturen. Alkalische  $Na_2S_2O_4$ -Lösung als  $O_2$ -Absorptionsmittel.

Behandlung der Hefesuspension vor dem Zusetzen der Zuckerlösung:

1. 30 Min. Schütteln mit strömender Luft.
  2. 30 Min. Schütteln mit strömendem  $N_2$ .
  3. 30 Min. Schütteln mit strömendem  $N_2$ , 60 Min. Schütteln in ruhender  $N_2$ -Atmosphäre.
  4. 30 Min. Schütteln mit strömendem  $N_2$ , 120 Min. Schütteln in ruhender  $N_2$ -Atmosphäre.
- 3 Parallelen.

Mengen Schwefeldioxyd, welche unter Umständen vom strömenden Stickstoff aus dieser Lösung mitgerissen werden können, wurden von der vor und nach dem elektrischen Rohrofen eingeschalteten konz. Kalilauge absorbiert (WIKÉN und RICHARD (74)). Uebrigens wurde dafür Sorge getragen, dass ein genügender Ueberschuss an Lauge in der Hyposulfitlösung vorhanden war.

Die Ergebnisse zweier Versuche, in welchen alkalische Natrium-hyposulfitlösung (nebst rotglühendem reduziertem Kupfer) zur  $O_2$ -Absorption diente, gehen aus Abb. 5 und 6 hervor. Es ist ersichtlich, dass sich das aus jungen (18 Stunden alten) Kulturen gewonnene Hefematerial bezüglich des  $N_2$ - bzw.  $O_2$ -Effekts gleich wie in den Versuchen mit alkalischer Pyrogallollösung verhielt. Die Geschwindigkeit der Vergärung der Glucose unter anaeroben Bedingungen nimmt mit wachsender Dauer der  $N_2$ -Behandlung stark ab. Für die mit Stickstoff während 30 Min. behandelte Hefesuspension konnte in Luft praktisch die gleiche Gärgeschwindigkeit festgestellt werden wie für die Suspension, welche in strömender Luft geschüttelt worden war (Abb. 5, Kurve 3 bzw. 1). Die Kohlendioxydbildung betrug in diesen Fällen in der approximativ linearen Gärphase 12,9 bzw. 13,7 ml/300 mg feuchte Hefe und Stunde. Der entsprechende Wert für die anaerobe Suspension (Abb. 5, Kurve 2) war 10,0 ml. Zum vollständigen Verbrauch der Glucose waren 280 bzw. 260 und 360 Min. nötig (Abb. 5, Kurve 3 bzw. 1 und 2).

Versuche mit Zellmaterial aus jungen und alten Kulturen in Argon bzw. Luft und Argon-Luft-Gemisch.

Bekanntlich kann gasförmiger, elementarer Stickstoff von zahlreichen aeroben und anaeroben, frei lebenden und symbiotischen, kohlenstoffheterotrophen und photoautotrophen Mikroorganismen zur Synthese der Zellsubstanz verwertet werden (siehe die kritische Uebersicht von WILSON im Lehrbuch von WERKMAN und WILSON (70) S. 473 ff.). Um sicher festzustellen, dass die Versuchsergebnisse, welche wir mit gewaschenem Zellmaterial aus jungen Kulturen der „Fendant“-Hefe erhalten haben, nicht auf eine Interferenz zwischen dem physiologisch nicht indifferenten Stickstoff und den Hefezellen im Sinne einer  $N_2$ -Bindung zurückzuführen sind, führten wir einige Experimente mit Argon durch. Dieses Gas kann wohl mit grosser Wahrscheinlichkeit als biologisch indifferent betrachtet werden (vgl. PORTER (52) S. 832, WERKMAN und WILSON (70) S. 483 ff.). Zur Entfernung der letzten  $O_2$ -Spuren diente (nebst rotglühendem



reduziertem Kupfer) alkalische Pyrogallollösung. Das Zellmaterial stammte aus 18 bzw. 20 und 66 bzw. 68 Stunden alten Kulturen. Die Ergebnisse liegen in Abb. 7 und 8 vor. Sie bestätigen eindeutig die Resultate der in Stickstoff bzw. Luft und Stickstoff-Luft-Gemisch ausgeführten Versuche, wie wir sie oben beschrieben und erläutert haben. Die aus jungen Kulturen gewonnenen Zellen zeigten unter streng anaeroben Bedingungen (Ar-Atmosphäre) eine wesentlich kleinere Gärgeschwindigkeit als in Luft (Abb. 7a und 8a, Kurve 2 bzw. 1). Die durch Ar-Begasung während 40 Min. hervorgerufene

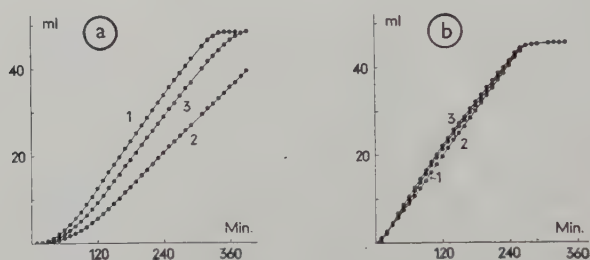


Abb. 7. Die Vergärung der Glucose in Luft bzw. Argon und Luft-Argon-Gemisch. Alkalische Pyrogallollösung als  $O_2$ -Absorptionsmittel.

Behandlung der Hefesuspension vor dem Zusetzen der Zuckerlösung:

1. 40 Min. Schütteln mit ruhender Luft.
2. 40 Min. Schütteln mit strömendem Ar.
3. 40 Min. Schütteln mit strömendem Ar, Zusatz von 2 ml Luft.

- a. Zellmaterial aus 18 Stunden alten Kulturen.
- b. Zellmaterial aus 66 Stunden alten Kulturen.

2 Parallelen.

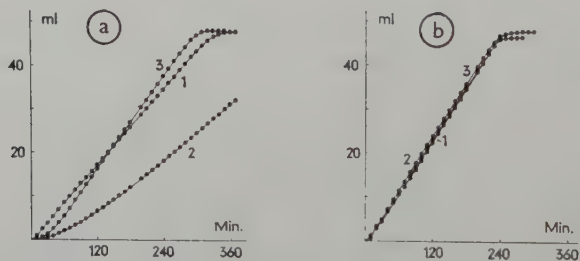


Abb. 8. Die Vergärung der Glucose in Luft bzw. Argon und Luft-Argon-Gemisch. Alkalische Pyrogallollösung als  $O_2$ -Absorptionsmittel.

Behandlung der Hefesuspension vor dem Zusetzen der Zuckerlösung:

1. 40 Min. Schütteln mit ruhender Luft.
2. 40 Min. Schütteln mit strömendem Ar.
3. 40 Min. Schütteln mit strömendem Ar, Zusatz von 2 ml Luft.

- a. Zellmaterial aus 20 Stunden alten Kulturen. Hefetrockensubstanz pro Gärkolben = 55,7 mg.
- b. Zellmaterial aus 68 Stunden alten Kulturen. Hefetrockensubstanz pro Gärkolben = 71,0 mg.

2 Parallelen.



Gärungshemmung wird in Anwesenheit von ca. 0,7 Volumen-% Sauerstoff in der Gasphase vollständig (Abb. 8a, Kurve 3) oder zum grössten Teil (Abb. 7a, Kurve 3) aufgehoben. Beim Zellmaterial aus den alten Hefekulturen konnten diese Effekte nicht nachgewiesen werden (Abb. 7b und 8b).

Versuche mit Zellmaterial aus sehr alten Kulturen in Stickstoff bzw. Luft.

Das Zellmaterial wurde aus Kulturen geerntet, welche 96 bzw. 144 und 192 Stunden alt waren. Die Versuchsergebnisse gehen aus Abb. 9 hervor. Die in Stickstoffatmosphäre gemessenen Gargeschwindigkeiten betrugen im annähernd linearen Abschnitt der

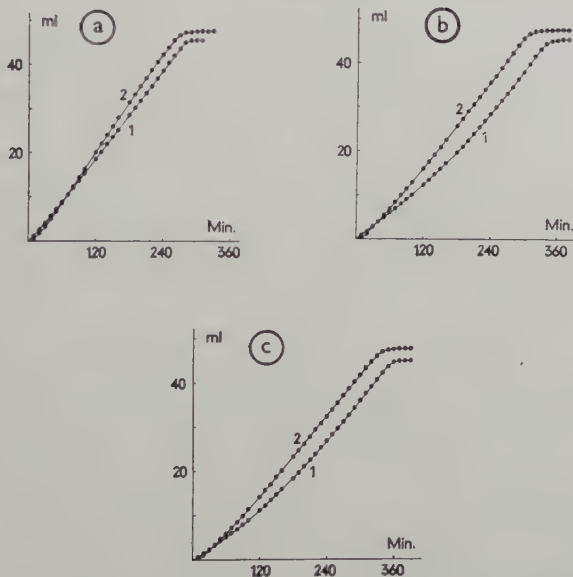


Abb. 9. Die Vergärung der Glucose in Luft bzw. Stickstoff. Alkalische Pyrogallollösung als  $O_2$ -Absorptionsmittel.

Behandlung der Hefesuspension vor dem Zusetzen der Zuckerlösung:

1. 40 Min. Schütteln mit ruhender Luft.
2. 40 Min. Schütteln mit strömendem  $N_2$ .
  - a. Zellmaterial aus 96 Stunden alten Kulturen.
  - b. Zellmaterial aus 144 Stunden alten Kulturen.
  - c. Zellmaterial aus 192 Stunden alten Kulturen.

2 Parallelen.

Gärkurven 11,3 bzw. 9,8 und 9,3 ml/300 mg feuchte Hefe und Stunde (Abb. 9a bzw. b und c, Kurve 2). Zur vollständigen Vergärung des Zuckers brauchten die Zellen 300 bzw. 340 und 370 Min. Die Gesamtvolumina des bei beendeter Gärung entwickelten

Kohlendioxyds waren 47,8 bzw. 47,7 und 47,8 ml. In Luft konnten für die entsprechenden gärenden Systeme totale Volumenvergrösserungen von nur 45,6 bzw. 45,5 und 45,0 ml festgestellt werden. Das Defizit von durchschnittlich 2,4 ml kann *a priori* auf verschiedene Prozesse in den ruhenden Hefezellen zurückgeführt werden. Beispielsweise könnte es dadurch entstanden sein, dass die Hefe unter aeroben Bedingungen einen grösseren Teil der Glucose unter Bildung nicht vergärbare Kohlehydrate umgesetzt hat als in sauerstofffreier Stickstoffatmosphäre. In diesem Zusammenhang sei an das Vorkommen von Trehalose als Reservekohlehydrat bei verschiedenen Hefen erinnert (KOCH und KOCH (31), ROBISON und MORGAN (56), VEIBEL (64), KLUYVER und VAN ROOSMALEN (30), STEINER und CORI (58), TANRET (60, 61), MYRBÄCK u. Mitarb. (10, 43, 44), NILSSON u. Mitarb. (45, 47), etc.). Dieses Disaccharid, das häufig in beträchtlichen Mengen in den Hefezellen vorliegt, wird unter Umständen bei der „Eigenatmung“ bzw. „Selbstgärung“ nicht oder nur äusserst langsam verbraucht, trotzdem die betreffenden Zellen über ein Enzymsystem zur Dissimilation des Zuckers verfügen (zugesetzte Trehalose wird vergoren). Wahrscheinlicher ist aber, dass das Defizit auf eine O<sub>2</sub>-Aufnahme durch die sehr alte Hefe zurückzuführen ist. Wie wir in einer weiteren Veröffentlichung noch ausführen werden (vgl. auch WIKÉN und RICHARD (75)), beträgt diese je nach dem Alter der zur Gewinnung des Zellmaterials verwendeten Kulturen 0,7- 1,3 ml 300 mg feuchte Hefe und Stunde (25° C., 72 cm Hg). Zu diesen Werten für die O<sub>2</sub>-Aufnahme der „Fendant“- Hefe ist allerdings zu bemerken, dass sie in Gefässen gemessen wurden, welche wir speziell entwickelt haben, um bei Mikroorganismen, die durch sehr kräftige Gärung und minimale O<sub>2</sub>-Atmung gekennzeichnet sind (Geschwindigkeit der CO<sub>2</sub>-Bildung  $\gg$  Geschwindigkeit der O<sub>2</sub>-Aufnahme), die letzte nachweisen und quantitativ bestimmen zu können. In diesen „Atmungskolben“, welche wir in einem separaten Artikel beschreiben werden, wird das Kohlendioxyd praktisch augenblicklich von Kalilauge absorbiert, während es sich in den „Gärkolben“, welche in der vorliegenden Arbeit zur Verwendung kamen (vgl. WIKÉN und RICHARD (74)), in der Gasphase anreichert. Bei einem pH-Wert der Hefesuspension von ca. 5 wird mit steigendem Partialdruck des Kohlendioxyds gemäss BRANDT (3) die in den Zellen gebundene Menge dieses Gases wesentlich erhöht. Die Messungen der Geschwindigkeit der O<sub>2</sub>-Afnahme wurden mithin unter ganz anderen Bedingungen be-

züglich des  $\text{CO}_2$ -Factors vorgenommen als die Gärversuche. Immerhin dürfen wir annehmen, dass die  $\text{O}_2$ -Atmung grössenordnungsmässig die gleiche ist in den „Gärkolben“ (hohe  $\text{CO}_2$ -Konzentration) wie in den „Atmungskolben“ ( $\text{CO}_2$ -Konzentration praktisch gleich Null), da man trotz der ausserordentlich raschen  $\text{CO}_2$ -Absorption in den letzten jedoch mit dem Vorhandensein kleiner Mengen dieser Verbindung in den Zellen rechnen muss (vgl. BRANDT (3)).

Unter der plausiblen Annahme, dass das im vorliegenden Versuch verwendete Hefematerial aus sehr alten Kulturen eine  $\text{O}_2$ -Atmung von durchschnittlich 1 ml/300 mg feuchte Hefe und Stunde aufweist, sollte das Defizit nach 120 bzw. 180, 240, 300 und 360 Min. ca. 2 bzw. 3, 4, 5 und 6 ml betragen. Die Unterschiede, welche zwischen den anaeroben und aeroben Hefesuspensionen experimentell festgestellt wurden (Abb. 9a - c, Kurve 2 bzw. 1), lassen sich grösstenteils durch diese  $\text{O}_2$ -Aufnahme erklären. Das aus sehr alten „Fendant“-Kulturen gewonnene Zellmaterial zeigt mithin praktisch keinen PASTEUR-MEYERHOF-Effekt.

Die oben beschriebenen und erörterten Versuchsergebnisse können folgendermassen kurz zusammengefasst werden:

Die Geschwindigkeit der Vergärung der Glucose durch ruhende Zellen der auxoautotrophen Kulturweihefe „Fendant“ aus jungen Kulturen ist vom gasförmigen elementaren Sauerstoff abhängig, indem sie in Luft sowie in Stickstoff und Argon mit einem geringen Gehalt an Sauerstoff wesentlich grösser ist als in sauerstofffreiem Stickstoff bzw. Argon. Das Gärvermögen dieses Zellmaterials nimmt kräftig ab bei zunehmender Dauer der  $\text{N}_2$ - bzw. Ar-Behandlung. Die so hervorgerufene Gärungshemmung kann durch Zusatz sehr kleiner  $\text{O}_2$ -Mengen vollständig oder zum Teil aufgehoben werden. Beim „Fendant“-Material, welches aus sehr alten Kulturen oder solchen mittleren Alters stammt, kann dieser Einfluss der  $\text{N}_2$ - bzw. Ar-Begasung und des Zusatzes kleiner  $\text{O}_2$ -Quantitäten nicht oder nur in geringem Ausmasse festgestellt werden. Die Geschwindigkeit der Vergärung der Glucose durch ruhende Zellen aus alten „Fendant“-Kulturen ist vom gasförmigen elementaren Sauerstoff praktisch unabhängig. Ein PASTEUR-MEYERHOF-Effekt lässt sich weder bei Zellmaterial aus jungen, noch bei solchem aus alten Kulturen nachweisen. Bei Zellen aus sehr alten Kulturen liegt eine Andeutung dieses Effekts vor. Die beim „Fendant“-Material verschiedenen Alters festgestellte  $\text{O}_2$ -Atmung ist sehr klein. Ihre Geschwindigkeit beträgt höchstens 5—10 % der maximalen Gärgeschwindigkeit.

Wesentliche Unterschiede scheinen somit zu bestehen zwischen dem Zymasesystem der aus jungen Kulturen geernteten „Fendant“-Zellen und demjenigen der Zellen, welche aus alten Kulturen der gleichen Hefe stammen. Die Zymase der ersten Zellen enthält eine oder mehrere für die Leistungsfähigkeit des Enzymsystems unerlässliche Komponenten, welche in zuckerfreien Medien unter streng anaeroben Bedingungen ( $O_2$ -freie  $N_2$ - bzw. Ar-Atmosphäre) in kurzen Zeitspannen ( $< 40$  Min.) reversibel, nach grösseren Zeitspannen ( $> 40$  Min.) auch irreversibel inaktiviert werden. Die reversible Inaktivierung lässt sich in Anwesenheit von ca. 1 Volumen-% Sauerstoff in der Gasphase rückgängig machen. Wie wir in einer weiteren Publikation näher beschreiben werden, kann dieser günstige Effekt bereits bei  $O_2$ -Konzentrationen von 0,01 bis 0,05 Volumen-% eindeutig nachgewiesen werden (vgl. WIKÉN und RICHARD (75)). Die Zymase des Zellmaterials aus alten „Fendant“-Kulturen zeigt unter streng anaeroben Bedingungen praktisch die gleiche Leistungsfähigkeit wie in Luft oder Gemischen von Stickstoff bzw. Argon mit kleinen Mengen Sauerstoff.

Schliesslich sei nochmals hervorgehoben, dass in sämtlichen Versuchen die Behandlung der Hefesuspensionen mit Luft bzw. Stickstoff oder Argon (strömende oder ruhende Atmosphäre) während verschiedener Zeitspannen vor dem Einkippen der Glucoselösung stattfand. Unmittelbar vor dem Beimischen des Zuckers erfolgte gegebenenfalls auch das Zusetzen der kleinen Luftmengen. Die Gärungen wurden sodann in Luft, sauerstofffreiem Stickstoff oder Argon bzw. Gemischen dieser Gase mit geringen Quantitäten Luft durchgeführt. In späteren Experimenten kamen synthetische Gemische von Stickstoff und Sauerstoff zur Verwendung (vgl. WIKÉN und RICHARD (75)).

Diese Arbeit ist ein Teil der Untersuchungen über die Physiologie der Weinhefen, welche von der Abteilung für Landwirtschaft des Eidg. Volkswirtschaftsdepartements aus dem Weinbaufonds unterstützt werden. Den zuständigen Behörden sprechen wir unseren herzlichen Dank aus. Die Ueberlassung der Kulturweinhefe „Fendant“ verdanken wir den Herren Professor Dr F. KOBEL und Dr H. LÜTHI, Eidg. Versuchsanstalt für Obst-, Wein- und Gartenbau, Wädenswil, Fräulein R. BUCHMANN und Fräulein A. MOHLER danken wir bestens für sorgfältige Assistenz.

### S u m m a r y.

In earlier as well as recent investigations on the significance of oxygen in regulating growth and multiplication, respiration and



fermentation (dissimilation of carbohydrates with and without oxygen uptake, respectively), thiamine and cytochrome content of yeasts, usually pure nitrogen or oxygen and ordinary or synthetic air (1 volume of oxygen + 4 volumes of nitrogen) were used as the atmosphere above the surface of the cell suspensions or for flushing and agitating the liquid phase.

The present study deals with the effect of air, oxygen-free nitrogen and argon as well as mixtures of the latter gases with very small amounts of oxygen (0.01-1 per cent by volume) upon the fermentation of glucose by resting cells of the auxo-autotrophic tame wine yeast *Fendant* grown for 18-20 (young cultures), 66-72 (old cultures), and 96-192 (very old cultures) hours, respectively, in a synthetic nutrient solution containing glucose, vitamin-free casein hydrolysate, potassium citrate buffer and mineral salts. After harvesting the yeast from the aerobic submerged growth cultures (shallow layers in conical Fernbach flasks incubated at 20° C. and shaken three times a day), the cells were washed by suspending and centrifuging twice in distilled water and once in the sodium succinate-succinic acid buffer solution used in the fermentation experiments (*cf.* below). The nitrogen and argon used for flushing the fermentation vessels were freed from oxygen by passing through alkaline solutions of pyrogallol or sodium hyposulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), washing with conc. sulfuric acid and potassium hydroxide and, subsequently, leading over reduced copper at 400-450° C. and, finally, washing with conc. potassium hydroxide and distilled water. The yeast suspensions and glucose solutions were prepared with 0.16 M sodium succinate-succinic acid buffer solution at pH 4.9.

The carbon dioxide production by the resting cell material prepared as described above was measured by means of the volumetric method developed by VON EULER, MYRBÄCK, NILSSON and ALM (46). The fermentation flasks contained 2.0 ml of yeast suspension in the main compartment and 1.0 ml of glucose solution in the side bulb. This corresponds to 300 mg of washed yeast (wet weight) and 200 mg of anhydrous sugar. The flasks were shaken at a rate of 100 complete oscillations per minute. For further experimental details we refer to WIKÉN and RICHARD (74).

The results reported in the present work prove conclusively the dependence of the metabolic activity of the yeast cells from young cultures on the duration of flushing with nitrogen or argon before the addition of glucose to the cell suspensions, the rate of carbon



dioxide production in the approximately linear phase of fermentation being decreasing considerably with increasing flushing times. Thus, in one experiment (Fig. 2) the rate of fermentation amounted to 11.9, 5.9, 4.3 and 3.3 ml of carbon dioxide per 300 mg of wet weight of yeast per hour after shaking the suspensions with air for 40 minutes, and with oxygen-free nitrogen for 40, 40 + 20, and 40 + 60 minutes, respectively. It should be noted, that these fermentations were measured in an atmosphere of the same kind of gas as used for flushing the suspensions. In presence of 1 per cent by volume of oxygen in the gas phase on the other hand, the yeast suspensions flushed with nitrogen for 40 minutes produced 12.0 ml of carbon dioxide per 300 mg of wet weight of yeast per hour, thus showing the same rate of fermentation as the suspensions shaken with air. Further experiments confirmed that the inactivation of the yeast from young cultures brought about by flushing with nitrogen (Fig. 4, 5 and 6) or argon (Fig. 7a and 8a) is removed, as a whole or partly (depending on the length of flushing time) by very small amounts of oxygen introduced prior to the addition of glucose. As a matter of fact, a marked stimulating action is observed even at oxygen tensions amounting to 0.01 - 0.05 per cent by volume (*cf.* WIKÉN and RICHARD (75)).

Resting cells from old Fendant cultures show the effects described above only to a very small extent. In a typical experiment (Fig. 3) fermentation rates amounting to 12.4, 12.4, 11.7 and 11.6 ml of carbon dioxide per 300 mg of wet weight of yeast per hour were obtained after flushing the yeast suspensions with air for 40 minutes, and with oxygen-free nitrogen for 40, 40 - 20, and 40 + 60 minutes, respectively. For the cells fermenting the sugar, after flushing with oxygen-free nitrogen for 40 minutes, in an atmosphere of nitrogen containing 1 per cent by volume of oxygen, the corresponding value of carbon dioxide production was 12.5 ml. Further experiments confirmed that cell material from old Fendant cultures shows the same rate of fermentation irrespective of the kind of treatment (shaking with air, flushing with nitrogen or argon for various lengths of time) before the addition of sugar (Fig. 7b and 8b; *cf.* WIKÉN and RICHARD (75)).

It may be assumed that in resting cells from young Fendant cultures one or more essential components of the zymase system are inactivated on flushing with nitrogen or argon. The activity is restored, wholly or partly, through introduction of minute amounts

of oxygen prior to the addition of sugar, the inactivation induced under anaerobic conditions thus being partly reversible and partly irreversible.

In addition to the strongly developed fermentative metabolic system (in cells from young cultures dependent on small amounts of oxygen), the Fendant yeast has a very low respiration, the rate of oxygen uptake by intact cells amounting to at most 5 - 10 per cent of the maximum rate of carbon dioxide liberation.

As far as an inhibition of fermentation by gaseous oxygen is concerned the Fendant yeast grown as described above for 18 - 20 (young cultures) and 66 - 72 (old cultures) hours does not show the PASTEUR-MEYERHOF effect. With suspensions of cells from very old cultures (incubated for 96 - 192 hours) a lower mean rate of increase in gas volume is obtained under aerobic (air) than under anaerobic (oxygen-free nitrogen) conditions (Fig. 9 a - c). This is largely due to the consumption of gaseous oxygen. It, however, also indicates the occurrence of a small PASTEUR-MEYERHOF effect in these cells.

Thus, the auxo-autotrophic tame wine yeast Fendant is a strongly fermenting and slowly respiring organism lacking practically the mechanism of the PASTEUR-MEYERHOF reaction.

#### L i t e r a t u r.

1. M. A. AMERINE and M. A. JOSLYN, Table Wines. Berkeley 1951. - 2. K. BERNHAUER, Gärungschemisches Praktikum. Berlin 1939. - 3. K. BRANDT, Acta Physiol. Scand. **10**, Suppl. XXX, 1945. - 4. W. BRAUN und R. PFUNDT, Biochem. Z. **287**, 115, 1936. - 5. E. C. CANTINO, Amer. J. Bot. **36**, 95, 1949. - 6. C. H. CHIN, Nature **165**, 926, 1950. - 7. M. TH. J. CUSTERS, Onderzoekingen over het gistgeslacht *Brettanomyces*. Diss. Delft 1940. - 8. G. DE BECZE and A. J. LIEBMAN, Ind. Eng. Chem. **36**, 882, 1944. - 9. E. R. EBERSOLE, C. GUTTENTAG and P. W. WILSON, Arch. Biochem. **3**, 399, 1944. - 10. M. ELANDER and K. MYRBÄCK, Arch. Biochem. **21**, 249, 1949. - 11. L. ENEBO, L. G. ANDERSON and H. LUNDIN, Arch. Biochem. **11**, 383, 1946. - 12. B. EPHRUSSI et P. P. SLONIMSKI, C.R. Acad. Sci. Paris **230**, 685, 1950. - 13. H. EULER und P. LINDNER, Chemie der Hefe und der alkoholischen Gärung. Leipzig 1915. - 14. H. FINK und E. BERWALD, Biochem. Z. **258**, 141, 1933. - 15. H. FINK und F. JUST, Biochem. Z. **308**, 15, 1941. - 16. H. FINK und R. LECHNER, Biochem. Z. **286**, 83, 1936. - 17. H. FINK, R. LECHNER und E. HEINISCH, Biochem. Z. **278**, 23, 1935; **283**, 71, 1936. - 18. H. FINK und M. ROSS, Biochem. Z. **323**, 389, 1953. - 19. J. W. FOSTER, Chemical Activities of Fungi. New York 1949. - 20. H. HAEHN, Biochemie der Gärungen. Berlin 1952. - 21. F. HAYDUCK und H. HAEHN, Biochem. Z. **128**, 568, 1922. - 22. H. D. HOBERMAN and D. RITTENBERG, J. Biol. Chem. **147**, 211, 1943. - 23. R. HÖBER, Physical

- Chemistry of Cells and Tissues. Philadelphia 1945. – 24. A. F. HOLLEMAN, Lehrbuch der organischen Chemie. Berlin 1930. – 25. J. C. HOOGERHEIDE, Bijdrage tot de kennis van de reactie van Pasteur. Diss. Delft 1935. – 26. A. JÖRGENSEN and A. HANSEN, Micro-organisms and Fermentation. London 1948. – 27. M. A. JOSLYN, Mycopath. et Mycol. Appl. **5**, 260, 1951. – 28. W. KEMPNER, Biochem. Z. **257**, 41, 1933. – 29. W. KEMPNER und F. KUBOWITZ, Biochem. Z. **265**, 245, 1933. – 30. A. J. KLUYVER and F. L. W. VAN ROOSMALEN, Biochem. Z. **245**, 13, 1932. – 31. E. M. KOCH and F. C. KOCH, Science **61**, 570, 1925. – 32. F. KUBOWITZ, Biochem. Z. **274**, 285, 1934. – 33. F. KUBOWITZ und E. HAAS, Biochem. Z. **255**, 247, 1932. – 34. E. KÜSTER, Anleitung zur Kultur der Mikroorganismen. Leipzig 1921. – 35. F. LIPMANN, A Symposium on Respiratory Enzymes, 48. Madison 1942. – 36. H. LÜERS, Die wissenschaftlichen Grundlagen von Mälzerei und Brauerei. Nürnberg 1950. – 37. H. LUNDIN, Biochem. Z. **141**, 310, 342, 1923; **142**, 454, 463, 1923. – 38. H. LUNDIN, J. Inst. Brew. **56** (47, New Series), 17, 1950. – 39. G. MENZINSKY, Ark. f. Kemi (Stockholm) **2:1**, 1950. – 40. R. MEYER und K. PIETSCHMANN, Abderhaldens Handb. biol. Arbeitsmeth. **XII:2**, 1513, Berlin 1939. – 41. O. MEYERHOF, Biochem. Z. **162**, 43, 1925. – 42. O. MEYERHOF, A Symposium on Respiratory Enzymes, 3. Madison 1942. – 43. K. MYRBÄCK, Svensk Kem. Tidskr. **49**, 24, 1937. – 44. K. MYRBÄCK und B. OERTENBLAD, Biochem. Z. **288**, 329, 1936; **291**, 61, 1937; **292**, 230, 1937. – 45. R. NILSSON, Schweiz. Z. f. allg. Path. Bakt. **13**, 672, 1950. – 46. R. NILSSON, Meth. d. Fermentforsch. (hg. von Bamann und Myrbäck) **3**, 2150, Leipzig 1941. – 47. R. NILSSON and F. ALM, Acta Chem. Scand. **3**, 213, 1949. – 48. B. H. OLSON and M. J. JOHNSON, J. Bact. **57**, 235, 1949. – 49. C. OPPENHEIMER and K. G. STERN, Biological Oxidation. den Haag 1939. – 50. L. PASTEUR, C. R. Acad. Sci. Paris **52**, 1260, 1861; Etudes sur la bière. Paris 1876; Oeuvre de Pasteur **5**, 206. Paris 1928. – 51. R. PFUNDT, Biochem. Z. **291**, 237, 1937; **294**, 300, 1937. – 52. J. R. PORTER, Bacterial Chemistry and Physiology. New York 1946. – 53. J. RIBÉREAU-GAYON, S. LAFOURCADE et M. LAFON, Ind. Agricol. Alim. **67**, 477, 1950. – 54. J. RIBÉREAU-GAYON, E. PEYNAUD et S. LAFOURCADE, Ind. Agricol. Alim. **68**, 141, 1951. – 55. A. RIPPEL-BALDES, Grundriss der Mikrobiologie. Berlin 1952. – 56. R. ROBISON and W. T. J. MORGAN, Biochem. J. **22**, 1277, 1928; **24**, 119, 1930. – 57. E. SIMON, Arch. Biochem. **13**, 237, 1947. – 58. A. STEINER and C. F. CORI, Science **82**, 422, 1935. – 59. J. B. SUMNER and G. F. SOMERS, Chemistry and Methods of Enzymes. New York 1947. – 60. G. TANRET, C. R. Acad. Sci. Paris **192**, 1056, 1931. – 61. G. TANRET, Bull. Soc. Chim. biol. **13**, 598, 1931. – 62. K. TRAUTWEIN und J. WASSERMANN, Biochem. Z. **229**, 128, 1930. – 63. F. P. TREADWELL, Kurzes Lehrbuch der analytischen Chemie. Band II. Leipzig 1930. – 64. S. VEIBEL, Biochem. Z. **239**, 350, 1931; **252**, 305, 1932. – 65. G. WAGNER, Gasanalytisches Praktikum. Wien 1942. – 66. O. WARBURG, Biochem. Z. **189**, 350, 1927. – 67. O. WARBURG, Biochem. Z. **189**, 354, 1927. – 68. O. WARBURG und E. NEGELEIN, Biochem. Z. **193**, 334, 339, 1928; **200**, 414, 1928; **202**, 202, 1928. – 69. W. S. WARING and C. H. WERKMAN, Arch. Biochem. **4**, 75, 1944. – 70. C. H. WERKMAN and P. W. WILSON, Bacterial Physiology. New York 1951. – 71. J. WHITE and D. J. MUNNS, Wallerstein Lab. Comm. **14**, 199, 1951. – 72. T. WIKÉN and O. RICHARD, Antonie van Leeuwenhoek **17**, 209,

---

1951; Schweiz. Z. f. allg. Path. Bakt. **14**, 560, 1951. – 73. T. WIKÉN und O. RICHARD, *Antonie van Leeuwenhoek* **18**, 31, 293, 1952. – 74. T. WIKÉN und O. RICHARD, *Antonie van Leeuwenhoek* **19**, 279, 1953. – 75. T. WIKÉN und O. RICHARD, *Experientia* **9**, 417, 1953. – 76. F. WINDISCH, *Biochem. Z.* **246**, 332, 1932.

---

(From the National Institute of Public Health, Utrecht).

## ON THE OCCURRENCE AND THE SIGNIFICANCE OF PARACOLON BACTERIA IN THE NETHER- LANDS

by

**J. G. HEYL**

(Received March 5, 1954).

### INTRODUCTION.

Until fairly recently the term "paracolon bacteria" was generally applied to all coliform micro-organisms that failed to ferment lactose within 24 hours with formation of acid and in the presence or absence of gas. Needless to say, this criterion alone is not a sufficient basis for the identification of so large a group of no doubt important bacteria.

Especially in the last 10 years attempts have been made, mainly in America, to stimulate a further development of our knowledge concerning the group of the paracolon bacteria and to give a lead to further researches.

STUART *et al.* (1940) proposed that all slow lactose-fermenting bacteria belonging to the coli-aerogenes group should be labelled as "aberrant coliform" bacteria, with a sub-division into sub-groups, based — apart from an agreement with the principal biochemical and cultural characteristics of the coli-aerogenes group — mainly on the degree of gas formation during the fermentation of lactose in relation to external factors, such as time, temperature, etc.

The chief drawbacks of STUART's classification are that the features mentioned for differentiation are hard to perceive and that there is no room in this system for the group of "aberrant coliform" bacteria which do not ferment lactose; a difficulty that STUART himself also felt and underlined with the words: "The existence of a group of non-lactose-fermenting coliforms is recognized, but this group is not sufficiently well delineated for satisfactory discussion".

It will have been partly owing to these circumstances, that the above classification has found little response.



In a later publication by STUART *et al.* (1943), which attracted more attention, he recommended a classification of the paracolon bacteria founded on imvic-reactions as well as on retarded or negative lactose fermentation, which would also provide a better parallel to the coli-aerogenes group. BORMAN *et al.* (1944) proposed the assumption on this basis of a separate genus *Paracolobactrum*, the type species of which would be formed by *Paracolobactrum coliforme* (analogous to *E. coli*, imvic ++ — —), *Paracolobactrum aerogenoides* (analogous to *Aerobacter aerogenes*, imvic — — ++ ) and *Paracolobactrum intermedium* (analogous to intermediate types, imvic variable).

Though this proposal has been accepted by several investigators, it should be borne in mind that this typification is only a makeshift and does not solve the problem. In this way one may indeed delineate a few groups of coliform micro-organisms more or less sharply, but with respect to the vast majority of the intermediate types there remains a gap in our knowledge. The same objection applies, more or less, to similar attempts to differentiate so heterogeneous a group of micro-organisms on the strength of a few biochemical reactions, as made, for instance, by SEVITT (1945), who founded the division of his paracoli-cultures into 4 groups exclusively on the fermentation of sucrose, dulcitol and lactose, and by SCHWABACHER (1949), who based her typification on the fermentation of lactose, sucrose, dulcitol and salicin.

SCHAUB (1946, 1948) has the credit of having got round this difficulty by making the ability or inability to develop on a desoxycholate-citrate-thiosulphate medium the chief criterion for the differentiation of the cultures studied by her. This enabled SCHAUB to divide her paracoli-cultures into 2 main groups, *viz.*, those which do not grow on this coli-inhibiting medium and are therefore related to the coli-aerogenes group, and those which do and may be regarded as intermediate types, related to the Salmonella-Shigella-Proteus group. According to SCHAUB this second main-group, which could be further divided into 3 groups and some sub-groups on the basis of biochemical characteristics, comprises the true and more important paracolon-bacteria. The first main-group, including the genus *Paracolobactrum* created by BORMAN *et al.*, she regards as aberrant forms of the coli-aerogenes group.

Especially the preponderantly serological researches by EDWARDS *et al.* and by KAUFFMANN, too, have contributed greatly to a better

knowledge of the paracolon bacteria related to *Salmonella*, *Shigella* and *Proteus*.

EDWARDS (1947) studied a great number of cultures of *Salmonella arizona* and related types and showed that these bacteria are more closely related to the paracoli, within which group they form a fairly homogeneous sub-group for which he proposed the name of Arizona-group. In the same way KAUFFMANN (1947) removed *Salmonella ballerup* and related types from the *Salmonella*-group, including them in the paracolon bacteria under the name of Ballerup-group.

In his further studies of *Enterobacteriaceae* EDWARDS (1948) succeeded in making up another homogeneous group of paracolon bacteria, which received the name of Bethesda-group. This group must be regarded as intermediate between the *Escherichia*-group on one hand and the *Salmonella*-group on the other.

The *Alcalescens*-Dispar group, named after *Shigella alcalescens* and *Shigella dispar* is regarded by several investigators (KAUFFMANN 1949; FRANTZEN, 1950) as a sub-division of the coli-aerogenes group and classed among the *Escherichiae*, others (EWING, 1949) speak of anaerogenic coliform or paracolon bacteria. Biochemically this group behaves like types of BORMAN's genus *Paracolobactrum*; hence their inclusion in the coli-aerogenes group can certainly be defended; on other grounds, for instance on the basis of SCHAUB's criteria, one might class them as paracoli related to coli and intermediate between coli and *Shigella*.

Lastly, a few words may yet be said in this connection about the cultures Type 29911 and Type B 5 W. Type 29911 (STUART, 1943) as a species of a group of anaerogenic paracolon bacteria, separated by STUART *et al.* (1946) into a number of sub-groups, should be considered intermediate between *Proteus* and *Shigella*.

KAUFFMANN (1951) has proposed the name of Providence-group for these micro-organisms. Type 29911, closely related to *Proteus*, is identical with *Shigella wakefield*.

Type B 5 W, also studied thoroughly by STUART *et al.* (1949) forms a fairly homogeneous group of anaerogenic, immotile paracolon bacteria, which are the only *Enterobacteriaceae* that do not reduce nitrate and have therefore been named *Bact. anitratum* by SCHAUB and HAUBER (1948). The place of these micro-organisms among the *Enterobacteriaceae* has not yet been defined, from a biochemical point of view they might be regarded as related to the *Shigella*-group.

The classification of the above mentioned groups of bacteria might be represented schematically as follows: It goes without saying, that, on the analogy of the KAUFFMANN-WHITE scheme for serological typing in the *Salmonella* group, a serological differentiation based on O- and H-antigens for the paracoli-groups too has been the subject of extensive studies. Though important results have also been obtained in this respect, this problem has not as yet been completely solved.

A detailed discussion of this subject, for which we may refer to KAUFFMANN (1951), would exceed the scope of this introduction; the data may be briefly summarized as follows. Serologically too, the group of paracolon bacteria that, according to SCHAUB's classification, might be placed in the genus *Paracolobactrum* is so clearly related to the coli-group, that KAUFFMANN classes these types with the *Escherichiae* and they have been included in the antigenic scheme drawn up by him and his collaborators KNIPSCHILDT and VAHLNE.

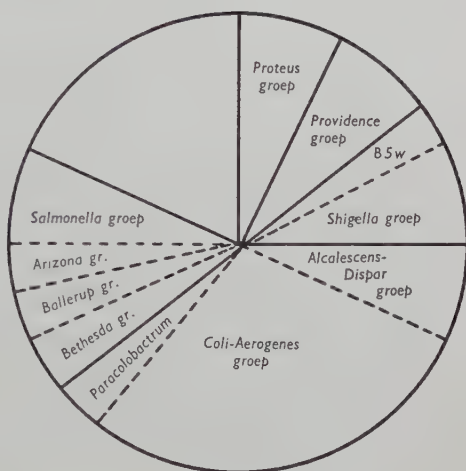
According to investigations by CHRISTENSON (1947), who found that 13 out of 16 cultures of *P. aerogenoides* were serologically related, this group might constitute a uniform whole.

On the basis of antigenic analysis BADER and KLEINMAYER (1952) thought they might assume a close relation between *P. coliforme* and *Shigella flexneri* type 6.

The fact that types of the *Salmonella*-group and of the Arizona-group have certain O- and H-antigens in common very clearly indicates that there is a close relation between these 2 groups.

Diphasic types have been described in the Arizona-group (WEST *et al.*, 1947; LE MINOR *et al.*, 1953).

The Ballerup-group is characterized by the possession of Vi-antigen and of O- and H-antigen related to *Salmonella* types (BRUNER, 1949).



Biochemical and serological researches have shown that the Bethesda-group constitutes a uniform whole in both respects, while the antigenic composition did not point to a close relation with other groups of the *Enterobacteriaceae* (MORAN *et al.*, 1949; EDWARDS *et al.*, 1948, GALBRAITH *et al.*, 1953).

FRANTZEN (1950, 1951) made a study of the Alcalescens-Dispar group and found that inclusion of this group in the *Escherichiae* is fully justified from a serological point of view.

In 131 cultures tested by him the O-antigen was either related to or identical with the O-antigen of *Escherichia*. This is a confirmation of KAUFFMANN's view on the place of the Alcalescens-Dispar-group.

Too little is known of the serology of the Providence-group as yet, to enable us to mention any definite views.

The serological work on STUART's B 5 W-group (*Bact. anitratum*), too, is still in an early stage. According to communications by STUART *et al.* (1949), the antigenic formula is very heterogeneous in biochemically identical strains.

One should not lose sight of the fact that the above groups do not form an uninterrupted series comprising the known *Enterobacteriaceae*, but are only more or less clearly defined units. Besides these units a number of probably pathogenic bacteria exists which can not be placed.

EDWARDS (1950), for instance, described a number of strains of intestinal bacteria with an antigenic structure pointing to relationship with the Salmonella-group and the Arizona-group, without it being possible to assign a definite place to them. FERGUSON (1947) found anaerogenic paracolon bacteria with the O-antigen of *Shigella sonnei*.

However, the expectation that a better insight into this matter will be gained in the future, is certainly justified.

#### MATERIAL AND RESULTS.

Numerous investigations now having sufficiently established the fact that many paracolon bacteria can be pathogenic for man, which pathogenicity mainly manifests itself in symptoms of gastro-enteritis (EDWARDS; BUTTIAUX and KESTELOOT, 1948; SCOTT, 1948) or as infections of the uro-genital tract (SCHAUB, 1946), it seemed worth while ascertaining whether these groups of bacteria also deserve more attention in our country.



Our interest was stimulated by the case of a three-year-old girl that had been admitted into hospital on account of secondary anaemia, which did not react to lactus ferrosus, lack of appetite and retarded growth. Clinical examination revealed a pyuria, which, from the case history, must have existed for some time. From the urine a gram-negative rod, which could be identified as *Paracolobactrum intermedium*, was repeatedly isolated. The culture was agglutinated by the serum from the patient.

When its sensitivity to antibiotics was determined, the micro-organism proved to be sensitive to streptomycin. After treatment with this antibiotic the urine soon became sterile and control cultures were negative. The reaction to lactus ferrosus was very favourable now and the anaemia as well as the other complaints soon disappeared.

From these facts it seemed fairly certain, that representatives of the group of paracolon bacteria belonging to group I of SCHAUB's classification (BORMAN's genus *Paracolobactrum*) may have some importance as pathogenic bacteria.

This was further borne out shortly afterwards by the case of a man with sepsis, from whose blood *Paracolobactrum aerogenoides* was isolated no less than three times.

The agglutination test with the patient's serum was positive. The application of streptomycin soon led to a lasting recovery. Repeated test-cultures were negative.

The identification of the isolated cultures was based almost exclusively on the comparison of cultural and biochemical properties with those of original test-cultures obtained from the U.S.A. A serological method of identification still involved too many difficulties to use it as a semi-routine technic.

In the course of this investigation, from 1950 onward, a total number of 458 cultures belonging to the *Paracolobactrum* group was isolated, viz., 177 cultures of *Paracolobactrum coliforme*, 171 cultures of *Paracolobactrum intermedium*, and 107 cultures of *Paracolobactrum aerogenoides*.

As it will appear from the following table, the occurrence of these micro-organisms was mainly restricted to the intestinal tract and the urogenital region.

As in certain tests for typhoid and paratyphoid fever the faeces and the urine of the patient are mixed before cultural testing, it was impossible in these cases to localize the *Paracolobactrum* cultures



TABLE I.

|                                  | faeces | urine | faeces<br>and<br>urine<br>mixed | total |
|----------------------------------|--------|-------|---------------------------------|-------|
| <i>Paracolobactrum coliforme</i> | 14.8%  | 44.4% | 29.6%                           | 88.8% |
| „ <i>intermedium</i>             | 5.1%   | 41.3% | 30.1%                           | 76.5% |
| „ <i>aerogenoides</i>            | 11.5%  | 11.5% | 49.2%                           | 72.2% |

thus obtained; these have therefore been entered in a separate column.

Our experiments showed that *Paracolobactrum intermedium* has a greater affinity for the urogenital tract than for the intestinal tract, a fact that to some extent also finds expression in this table.

It was often impossible to ascertain whether there actually was any aetiological connection between these micro-organisms isolated from the faeces and urine of patients and their complaints.

There may well be some connection between the presence of these paracolon bacteria in the stools and urine from patients, submitted for testing on typhoid, paratyphoid fever or dysentery, and the clinical picture (NELSON and DESMIN, 1951). A further inquiry into this matter is intended.

In several cases, however, as in the above instances, the probability of a causal relation between the complaints and the isolated micro-organisms could indeed be demonstrated.

No representatives of the Arizona-, Bethesda- and Ballerup-groups were ever isolated from patients in these years.

Although it would appear, therefore, that these bacteria are of no significance as pathogenic micro-organisms in our country, it should be noted that the occurrence of bacteria belonging to these groups in man has been established in France and Italy. Besides, bacteria of the Arizona-group were occasionally isolated here from imported egg-powder. EDWARDS *et al.* (1947) also repeatedly isolated these micro-organisms from egg-powder. In this connection it may also be worth mentioning that a bacterium related to this group was recently isolated from pus from a patient who had fallen into a dirty drain after being wounded in a road-accident. Although the occurrence of a micro-organism belonging to this group in purulent wounds seems strange, this possibility has already been confirmed

by BUTT and MORRIS (1952), who isolated a paracolon bacterium of the Arizona-group from pus from an otitis.

The occurrence in this country of bacteria belonging to the Alcalescens-Dispar-group in man appears to be a rare phenomenon. In the course of this work not a single strain belonging to this group was isolated. However, it may be assumed that the *Shigella* strain Katwijk formerly isolated and described by SCHOLTENS (1940) belongs to the dispar types in this group.

In the period of these studies no data were obtained concerning the Providence-group either.

With respect to STUART's group B 5 W (*Bact. anitratum*) the situation is quite different. The pathogenicity of this bacterium has been sufficiently demonstrated. Although *Bact. anitratum* is mainly to be found in urine, its occurrence is by no means restricted to the intestinal and urogenital tracts, as is almost exclusively the case with many paracolon bacteria (STUART, 1949; FERGUSON, 1950).

In Denmark BROOKE (1951) succeeded 87 times in isolating this bacterium from patients, over 60% from the urogenital tract and almost 20% from abscesses, pus etc.

In the course of our investigations this micro-organism was found 51 times in material submitted for testing; of this 47% came from the urogenital tract and over 20% from pus, punctates etc. As regards the remaining cultures, these confirm BROOKE's detailed statements, that *Bact. anitratum* may occur anywhere in the body and can cause local infections. No doubt this micro-organism needs more attention in the diagnosis of pathogenic bacteria.

The results of this investigation into the occurrence of paracolon bacteria in the Netherlands seem to justify the opinion that these bacteria invite attention both from a bacteriological and from a pathological point of view and that a closer study of these micro-organisms on a broader basis is certainly justified.

### S u m m a r y.

After an introductory survey, in which, among other things the taxonomy of the various groups of known paracolon bacteria was discussed, we investigated the occurrence of these groups in the Netherlands on the basis of our own researches. It appeared that it was chiefly the aberrant coliform bacteria included in BORMAN's genus *Paracolobactrum* and STUART's B 5 W group (*Bact. anitratum*) which could be qualified as pathogenic bacteria.

## References.

- BADER, R. E. and KLEINMAYER, H., 1952. *Z. f. Hyg.* **135**, 82.  
BORMAN, E. K., STUART, C. A. and WHEELER, K. M. 1944. *J. Bact.* **48**, 351.  
BROOKE, M. S. 1951. *Acta Path. Microb. Scand.* **28**, 338.  
BRUNER, D. W., EDWARDS, P. R. and HOPSON, A. S. 1949. *J. Inf. Dis.* **85**, 290.  
BUTT, E. and MORRIS, J. E. 1952. *J. Inf. Dis.* **91**, 282.  
BUTTIAUX, R. and KESTELOOT, A. 1948. *Ann. Inst. Past.* **75**, 379.  
CHRISTENSEN, W. B. 1947. *J. Bact.* **53**, 317.  
EDWARDS, P. R. 1945. *J. Bact.* **49**, 513.  
EDWARDS, P. R., WEST, M. G. and BRUNER, D. W. 1947. *Kent. Agr. Exp. Stat. Bull.* **499**; *J. Inf. Dis.* **81**, 19.  
EDWARDS, P. R., WEST, M. G. and BRUNER, D. W. 1948. *J. Bact.* **55**, 711.  
EDWARDS, P. R. and WEST, M. G. 1950. *J. Inf. Dis.* **87**, 184.  
EWING, W. H. 1949. *J. Bact.* **58**, 497.  
FERGUSON, W. W. and HENDERSON, N. D. 1947. *J. Bact.* **54**, 179.  
FERGUSON, W. W. and ROBERTS, L. F. 1950. *J. Bact.* **59**, 171.  
FRANTZEN, E. 1950. *Acta Path. Microb. Scand.* **27**, 236.  
FRANTZEN, E. 1951. *Acta Path. Microb. Scand.* **28**, 103.  
GALBRAITH, E. L. and CROSSLEY, V. M. 1935. *Can. J. Publ. Health* **44**, 172.  
KAUFFMANN, F. 1947. *Acta Path. Microb. Scand.* **24**, 242.  
KAUFFMANN, F. 1949. *Acta Path. Microb. Scand.* **26**, 879.  
KAUFFMANN, F. 1951. *Enterobacteriaceae*.  
MINOR, L. LE, EDWARDS, P. R. and FOSSAERT, H. 1953. *Ann. Inst. Past.* **84**, 1056.  
MORAN, A. B. and BRUNER, D. W. 1949. *J. Bact.* **58**, 695.  
NELSON, R. M. and DESMIN, C. 1951. *Proc. Soc. Exp. Biol. Med.* **76**, 737.  
SCHWABACHER, H. 1949. *J. Path. Bact.* **61**, 63.  
SCHAUB, I. G. 1946. *J. Lab. Clin. Med.* **31**, 958.  
SCHAUB, I. G. 1948. *Bull. John Hopkins Hosp.* **83**, 367.  
SCHAUB, I. G. and HAUBER, F. D. 1948. *J. Bact.* **56**, 379.  
SCHOLTENS, R. 1940. *Nederl. Tijdschr. v. Geneesk.* **84**, 4613.  
SCOTT, T. F. M., DAVIS, H. J., CORIELL, L. L. and BOLTIJES, B. H. 1948. *Ann. J. Dis. Child.* **75**, 426.  
SEVITT, S. 1945. *J. Hyg.* **44**, 37.  
STUART, C. A., MICKLE, F. C. and BORRAN, E. K. 1940. *Am. J. Publ. Health* **30**, 499.  
STUART, C. A., WHEELER, K. M., RUSTIGIAN, R. and ZIMMERMAN, A. 1943. *J. Bact.* **45**, 101.  
STUART, C. A., WHEELER, K. M. and MCGANN, V. 1946. *J. Bact.* **52**, 431, 519.  
STUART, C. A., GALTON, M. M. and MCGANN, V. 1948. *J. Bact.* **56**, 411.  
STUART, C. A., FORMAL, S. and MCGANN, V. 1949. *J. Inf. Dis.* **84**, 235.  
WEST, M. G., EDWARDS, P. R. and BRUNER, D. W. 1947. *J. Inf. Dis.* **81**, 24.

(From the National Institute of Public Health and the Clinic for Small Animals of the State University, Utrecht).

## *SALMONELLA ISRAEL* IN A DOG

by

A. CLARENBURG, G. H. B. TEUNISSEN and J. VAN DER VLERK

(Received February 13, 1954).

*Salmonella israel* was examined for the first time by P. R. EDWARDS (U.S.A.) in 1953. The culture had been isolated from the stools of a normal human carrier in Israel (personal communication). Shortly afterwards, April 1953, an identical culture, isolated from the stools of an adult female, was examined independently by CLARENBURG, MARSEILLE and VINK (1). It is quite a coincidence that in June 1953 the same *Salmonella* type was isolated from the faeces of a 3-year-old female dog. There was no connection whatever between the infection of this dog and that of the above-mentioned woman. The dog had been suffering for a considerable time from relapsing diarrhoea.

### Laboratory examination.

The faeces of the dog were plated directly and also after enrichment in tetrathionate broth modified by KAUFFMANN on to brilliant green-phenol red agar. Only with the enrichment method were *Salmonella* colonies found.

The biochemical and serological characteristics of the isolated type were the same as those of the culture isolated from the aforesaid woman.

Serologically the type had the formula: 9, 12: e,h-e,n,z<sub>15</sub>. Cross-absorptions showed that the O antigen was identical with the O antigen of *S. dublin*, the H antigen of phase 1 (e,h) was identical with the H antigen of *S. eastbourne*, phase 1, and the H antigen of phase 2 (e,n,z<sub>15</sub>) was identical with the H antigen of *S. san diego*, phase 2.

A few weeks later the dog was submitted to the Clinic for Small Animals for observation. No symptoms of disease were found now.

Notably the faeces were normal during the 3 weeks of observation. We performed a weekly examination of the faeces on the presence of *Salmonella* organisms and of the bloodserum on agglutinins. The result was that no *Salmonella* was found; the agglutination reactions of the bloodserum with O antigen and H antigen phase 2 of *S. israel* were 1 : 20 negative, with H antigen phase 1 of *S. israel* 1 : 80 positive.

### S u m m a r y.

*Salmonella israel* was isolated from the faeces of a 3-year-old dog suffering from relapsing diarrhoea. This is the first time that this *Salmonella* type has been found in animals.

### R e f e r e n c e s.

- CLARENBURG, A., MARSEILLE, A. and VINK, H. H. 1954. *Antonie van Leeuwenhoek* **20**, 319.
-



(From the National Institute for Public Health, Utrecht).

# ON THE PATHOGENESIS OF DIPHTHERIA

## III. ON THE FORMATION OF DIPHTHERIA TOXIN IN FRESH HUMAN SALIVA <sup>1)</sup>

by

A. TASMAN and L. SMITH

(Received March 19, 1954).

Recently TASMAN and SMITH (1953 *a*) published a detailed study of the formation of diphtheria toxin in sterilized human saliva. They showed that saliva is a suitable medium for *C. diphtheriae*, in which, under certain circumstances, diphtheria bacteria can produce comparatively large quantities of diphtheria toxin. This applies both to the well-known "P.W. 8" strain and to strains isolated from patients.

DOLD and WEIGMANN (1935) showed that most human saliva has an indubitable bactericidal effect on diphtheria bacteria. The inducing agent they called „Inhibine". Only fresh saliva, not previously inactivated by heating at 56° C. proved to have this property. The active component is very labile. Even when saliva is merely kept at room temperature for 1-2 days, its bactericidal effect is considerably reduced. When the originally very virulent diphtheria bacteria are grown for a prolonged period on „saliva-agar plates", they lose their virulence and cannot be stained by the Neisser and Gram method anymore nor do they ferment glucose. The saliva samples obtained from different volunteers varied greatly in their bactericidal effect.

BÖNICKE, REIF and ARNDT (1951) were able to confirm the results of DOLD and WEIGMANN (1935) and at the same time found that the bactericidal activity of the saliva of one and the same person fluctuated considerably in the course of 24 hours, the bactericidal effect of so-called "pre-breakfast" saliva, produced immediately after waking, being the greatest. They draw attention to the fact that in one's sleep the production of saliva is much smaller than when

<sup>1)</sup> Part I and II: Antonie van Leeuwenhoek 19, 135, 333, 1953.

one is awake. Consequently, part of the saliva produced in sleep remains in the oral cavity for a comparatively long time and is, so to speak, "condensed"; and as a result of this the bactericidal substances occur in a higher concentration in this "pre-breakfast" saliva than in the saliva produced in the course of the day. This bactericidal effect and its daily fluctuations were studied with respect to *Micrococcus aureus* and *Bac. subtilis*. They, too, point out the great lability of the efficient agent. DOLD (1940) also found this bactericidal effect in saliva taken under sterile conditions from the Gl. Submandibularis of dogs.

Little is known as yet about the nature of these "bactericidal" compounds. FLEMING (1922) believes that the effect is caused by lysozyme. BETHGE, SOEHRING and TSCHESCHE (1947) ascribed it to the formation of  $H_2O_2$ , but this was emphatically denied by BÖNICKE, REIF and ARNDT (1951), in view of the results of their experiments. BERGER (1952), however, was unable to find an "inhibine" for *C. diphtheriae* as described by DOLD and WEIGMANN (1935), nor could he ever demonstrate the presence of  $H_2O_2$  in human saliva.

THOMPSON and SHIBUYA (1946) stated that the Streptococci being present in human saliva (especially *Str. viridans*) should be responsible for the bactericidal effect of saliva against *C. diphtheriae*. This bactericidal effect should be antagonized by staphylococci.

As, in studying the pathogenesis of diphtheria, we aimed at the closest approximation to the pathological circumstances of the patients we now concentrated upon fresh, non treated human saliva.

Saliva samples were obtained from 70 children of ages ranging from 4—6. Part of these samples could be examined within 1—2 hours after production. Another part could be examined on the day of their production, but not until about 4 hours had elapsed after they had been collected. Lastly, a number of saliva samples were kept in the refrigerator ( $\pm 2^\circ C.$ ) for one night, owing to special circumstances.

The importance of the lapse of time between the production of the saliva and its examination will be discussed later.

One portion of each saliva sample was inoculated with the P.W.8 strain<sup>1)</sup> in its "fresh" state, i.e. without first being heated, another portion being first autoclaved. In addition, the amino-acids in

<sup>1)</sup> The P.W.8. strain used had been obtained from LEVINE (1949) of the Mass. Dept. Publ. Health, Mass., U.S.A.

each portion were determined by two-dimensional paper chromatography, in the manner described by TASMAN and SMITH (1953*b*).

All the saliva cultures were incubated at 34-35° C. for seven days, after which the quantities of diphtheria toxin that had been produced were determined by the method previously described (TASMAN and SMITH, 1953*a*). The results were as follows.

After inoculation with the P.W.8 strain and incubation during one week, it was evident that all the "fresh" saliva samples greatly inhibited the formation of diphtheria toxin<sup>1</sup>). After a week's incubation all the autoclaved saliva samples contained a considerably larger quantity of toxin than the non-autoclaved samples. However, with one or two exceptions, measurable quantities of toxin were also found in the non-autoclaved saliva samples after a week's growth. The quantities of toxin formed in these samples varied greatly.

No connection could as yet be established between the amino-acid pattern of the saliva and the quantity of diphtheria toxin formed in it ( see TASMAN and SMITH, 1953*b*).

In order to ascertain the quantities of diphtheria toxin that had been produced, every saliva toxin was diluted with saline and 0.2 ml of these dilutions was injected intracutaneously into a guinea-pig. The red spots on the skin of the guinea-pig were judged after two days and compared with a so-called "standard spot". This "standard spot" was caused by injecting intracutaneously 0.2 ml of a solution of a diphtheria toxin that had also been used as a control in previous investigations. The solution contained 0.0000058 ml of this toxin per 0.2 ml, this quantity of diphtheria toxin being called one "reaction dose". The quantities of toxin formed in the various saliva samples were expressed in number of reaction doses per ml of saliva compared with the "standard spot".

Table 1 gives the results obtained with 20 saliva samples taken at random, one portion of each sample being inoculated with the P.W.8 strain without being sterilized first, and another portion (of the same sample) after having been autoclaved. On an average the saliva inoculated after sterilization contains about 500 times as much toxin at the end of the incubation period as the saliva not previously autoclaved.

<sup>1</sup>) For convenience'sake the inhibitory effect on the formation of diphtheria toxin will henceforth be referred to as "inhibition" or "inhibitory effect".

TABLE 1.

Effect of previous heating on the formation of diphtheria toxin in human saliva after inoculation with the P.W.8 strain and one week's incubation at 34-35° C.

| No. of saliva sample | Number of "reaction doses" of diphtheria toxin per ml of saliva |                                          |
|----------------------|-----------------------------------------------------------------|------------------------------------------|
|                      | Saliva autoclaved before inoculation                            | Saliva not autoclaved before inoculation |
| 1                    | 10.000                                                          | 500                                      |
| 2                    | 175.000                                                         | 50                                       |
| 3                    | 50.000                                                          | 75                                       |
| 4                    | 6.500                                                           | 100                                      |
| 5                    | 5.000                                                           | 170                                      |
| 6                    | 10.000                                                          | 10                                       |
| 7                    | 145.000                                                         | 65                                       |
| 8                    | 5.000                                                           | 670                                      |
| 9                    | 1.450.000                                                       | 350                                      |
| 10                   | 7.000                                                           | 75                                       |
| 11                   | 50.000                                                          | 1.000                                    |
| 12                   | 65.000                                                          | 500                                      |
| 13                   | 100.000                                                         | 18                                       |
| 14                   | 70.000                                                          | 50                                       |
| 15                   | 50.000                                                          | 750                                      |
| 16                   | 75.000                                                          | 10                                       |
| 17                   | 6.500                                                           | 500                                      |
| 18                   | 50.000                                                          | 75                                       |
| 19                   | 65.000                                                          | 720                                      |
| 20                   | 5.000                                                           | 10                                       |
| average              | 120.000                                                         | 285                                      |

The results given in table 2 will show that the keeping of "fresh" saliva at  $\pm 2^\circ$  C. also results in a marked loss of inhibitory effect.

The second column of table 2 comprises 20 saliva samples taken at random and placed in the refrigerator for one night before the inoculation. The third column gives 20 other samples chosen in the same way, but inoculated about one hour after production. That even a few hours' standing at room temperature already greatly impairs inhibition will appear from a comparison of the data of the third column of table 1 with the corresponding figures of the third column of table 2. A difference of only three hours' standing at room temperature lowers the average toxin yield of "fresh" saliva from 285 to 157 reaction doses.

TABLE 2.

Effect of previous keeping of saliva samples on the production of diphtheria toxin with the P.W.8 strain after one week's incubation.

| No. of saliva sample | Number of 'reaction doses' of diphtheria toxin<br>per ml of saliva            |                                                                |
|----------------------|-------------------------------------------------------------------------------|----------------------------------------------------------------|
|                      | Saliva sample kept for 1<br>night at $\pm 2^{\circ}$ C. before<br>inoculation | Saliva sample inoculated<br>$\pm 1$ hour after col-<br>lection |
| 1                    | 5000                                                                          | 500                                                            |
| 2                    | 20                                                                            | 50                                                             |
| 3                    | 1000                                                                          | —                                                              |
| 4                    | 500                                                                           | 100                                                            |
| 5                    | 20                                                                            | 160                                                            |
| 6                    | 7000                                                                          | 10                                                             |
| 7                    | 140                                                                           | 60                                                             |
| 8                    | 1000                                                                          | 700                                                            |
| 9                    | 550                                                                           | 100                                                            |
| 10                   | 750                                                                           | 10                                                             |
| 11                   | 100                                                                           | 500                                                            |
| 12                   | 10                                                                            | 100                                                            |
| 13                   | 500                                                                           | 10                                                             |
| 14                   | 50                                                                            | —                                                              |
| 15                   | 1500                                                                          | 50                                                             |
| 16                   | 750                                                                           | 20                                                             |
| 17                   | 20                                                                            | 15                                                             |
| 18                   | —                                                                             | 500                                                            |
| 19                   | 1000                                                                          | 50                                                             |
| 20                   | 500                                                                           | 200                                                            |
| average              | 972                                                                           | 157                                                            |

As already stated, we intended our investigation to approximate as closely as possible to the conditions present in the diphtheria patient, and therefore we now studied the effect of keeping the saliva at  $34-35^{\circ}$  C. before inoculation on the subsequent formation of toxin.

A quantity of about 25 ml of saliva was obtained in about 15 minutes from each of three persons and was distributed over 7 erlenmeyer flasks of 10 ml. Every flask contained about 3 ml of saliva. Thereupon the contents of the first flask were immediately inoculated with the P.W.8 strain, and the contents of the other flasks after being kept for 1, 2, 3, 5, 10 and 24 hours respectively at  $34-35^{\circ}$  C.



All the samples thus inoculated were incubated for 7 days at 34-35° C., after which their toxin content was determined. The results are given in table 3.

TABLE 3.

Toxin production in saliva samples obtained from several persons, inoculated at once and after having been kept at 34-35° C., incubated for one week at 34-35° C.

| Saliva sample | Number of "reaction doses" of diptheria toxin per ml of saliva.<br>Saliva kept at 34-35° C. before the inoculation. |        |         |         |         |          |          |
|---------------|---------------------------------------------------------------------------------------------------------------------|--------|---------|---------|---------|----------|----------|
|               | inoculated at once                                                                                                  | 1 hour | 2 hours | 3 hours | 5 hours | 10 hours | 24 hours |
| W.            | 50                                                                                                                  | 100    | 1,000   | 1,700   | 9,700   | 17,500   | 50,000   |
| v.Rh.         | 10                                                                                                                  | 50     | 100     | 50      | 50      | 10       | —        |
| v.R.          | 10                                                                                                                  | 50     | 500     | 500     | 500     | 1,000    | 1,000    |

Indeed, it appeared that in this serial experiment, too, the quantity of toxin formed depended on the time during which the saliva samples had been kept at 34-35° C. before they were inoculated with the P.W.8 strain. In proportion with the interval of time the toxin yield increased. The results of this experiment are represented graphically in fig. 1. The quantities of toxin are here expressed

logarithmically.

For the saliva of "v.Rh" there is apparently an optimal time of two hours' keeping at 34-35° C. before inoculation, after which period the toxin yield drops again.

A very remarkable thing was the lasting effect of these comparatively small differences of time on the quantity of toxin formed, although after the inoculation the saliva was incubated for a week. These results of our experiments

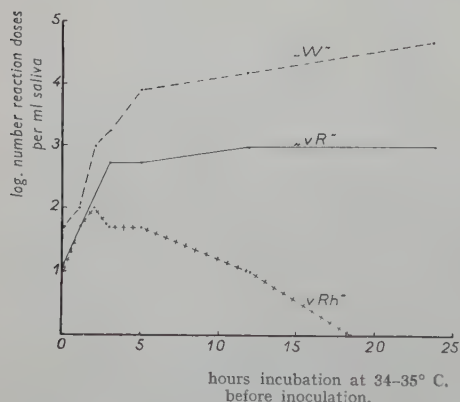


Fig. 1. Toxin production in saliva samples obtained from different persons, inoculated immediately and after previously incubation at 34-35° C. P.W. 8 strain; incubated for one week at 34-35° C.

led us to suppose that the inhibitory effect of saliva on the toxin producing capacity of diphtheria bacteria might have a lasting influence on the generations of diphtheria bacteria that developed during the incubation.

In his researches concerning bacterial variation and toxin production of diphtheria bacteria, AMIES (1948) obtained similar results.

At the same time the thought suggested itself, that once the "inhibitory effect" had worn off, the actual toxin formation would require a comparatively short space of time. Fresh saliva of a volunteer whose saliva showed but little inhibitory effect was inoculated with the P.W.8 strain and the toxin production after an incubation of respectively 30 minutes, 1, 2, 3 and 5 hours was then ascertained. Already after 3 hours' incubation a clear toxin reaction could be obtained with such a saliva sample!

Saliva was now obtained from two volunteers. These samples were each divided into seven equal parts, which were succesively inoculated with the P.W. 8 strain immediately after the saliva had been obtained and after having been kept at 34-35° C. for 30 minutes, and 1, 3, 5, 12 and 24 hours respectively. All these portions were then incubated at 34-35° C. for only 12 hours, after which they were examined for their toxin content. The results of this experiment are given in table 4 and represented graphically in figure 2.

TABLE 4.

Toxin formation in saliva samples of two volunteers, inoculated at once and after having been kept at 34-35° C. P.W.8 strain; 12 hours' incubation at 34-35° C.

| Saliva sample | Number of "reaction doses" of diphtheria toxin per ml of saliva.<br>Saliva kept at 34-35° C. before the inoculation. |        |        |         |         |          |          |
|---------------|----------------------------------------------------------------------------------------------------------------------|--------|--------|---------|---------|----------|----------|
|               | inoculated at once                                                                                                   | ½ hour | 1 hour | 3 hours | 5 hours | 10 hours | 24 hours |
| v.R.          | —                                                                                                                    | 0.5    | 0.5    | 0.5     | 1.6     | 1.6      | 1.0      |
| v.P.          | 0.3 <sup>1)</sup>                                                                                                    | 0.9    | 1.6    | 1.6     | 1.6     | 4.3      | 10.8     |

<sup>1)</sup> Quantities of diphtheria toxin smaller than one reaction dosis were judged by taking for the "standard spot" a quantity of toxin smaller than one reaction dose, *i.e.* one tenth of this.

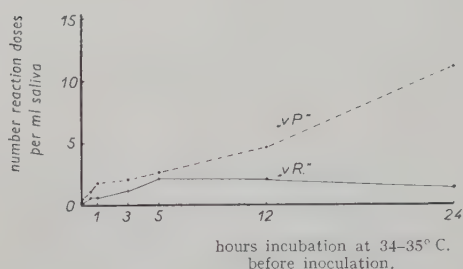


Fig. 2. Toxin production in saliva samples obtained from 2 persons; inoculated immediately and after having been kept at 34-35° C.; P.W.8 strain; incubated for 12 hours at 34-35° C.

The formation of toxin was repeatedly found to be much slower after inoculation into the saliva of volunteer "v.R." than after inoculation into the saliva of volunteer "v.P." under identical conditions. No toxin could be indicated in the saliva of "v.R.", when this was immediately inoculated with the P.W.8 strain and incubated for only 12 hours.

The presence of toxin could only be shown when the saliva had been kept at 34-35° C. during at least half an hour before the inoculation.

Finally, these experiments were repeated with diphtheria strains isolated from patients. For these experiments samples of the saliva of the same two persons, "v.R." and "v.P.", were always used.

The results did not differ essentially from those we got with the P.W. 8 strain. In these experiments, too, the inhibitory effect of the "v.R." saliva proved to be stronger and to last longer than that of the "v.P." saliva. It was, however, a striking fact, that "patient strains" required less time to neutralize this

TABLE 5.

Formation of diphtheria toxin in saliva of two volunteers, inoculated at once and after having been kept at 34-35° C. Different patient strains; 12 hours' incubation time.

| Saliva sample | No. Patient strain | Number of "reaction doses" of diphtheria toxin per ml of saliva. Saliva kept at 34-35° C. before the inoculation. |        |        |         |         |          |          |
|---------------|--------------------|-------------------------------------------------------------------------------------------------------------------|--------|--------|---------|---------|----------|----------|
|               |                    | inoculated at once                                                                                                | ½ hour | 1 hour | 3 hours | 5 hours | 12 hours | 24 hours |
| "v.R."        | 7358               | —                                                                                                                 | —      | 5      | 5       | 7       | 9        | 5        |
| "v.P."        | 7358               | —                                                                                                                 | 5      | 10     | 35      | 58      | 85       | 15       |
| "v.R."        | 7718               | —                                                                                                                 | —      | 3.3    | 5       | 10      | 5.3      | 6.4      |
| "v.P."        | 7718               | —                                                                                                                 | 5      | 15     | 50      | 83      | 34       | 34       |
| "v.R."        | 8035               | 1.7                                                                                                               | 1.7    | 1.7    | 1.7     | 6.7     | —        | —        |
| "v.P."        | 8035               | —                                                                                                                 | —      | 5      | 5       | 10      | 35       | 55       |

inhibitory effect than the P.W.8 strain, and could thus produce considerably larger quantities of toxin. Table 5 gives the results of these experiments.

An illustration of these results, those obtained with patient strain No 7718 are represented graphically in figures 3 and 4.

As it seems to us that it might be of special importance with respect to the diphtheria patient to know how much toxin could be formed in his saliva after this had been given only a short "rest" (e.g. overnight, during sleep), the quantities of toxin produced after the saliva had been kept from 0-5 hours at 34-35° C. are shaded in these two figures.

In these tests, too, there appears to be an optimal time of incubation before inoculation. This is especially evident in figure 4.

After previous experiments had already shown that comparatively large quantities of diphtheria toxin could be produced in a short time in fresh saliva kept at 34-35° C. for only a few hours before inoculation, the incubation time was finally reduced from 12 hours

to 9 hours in the last experiment. Samples of saliva obtained from several volunteers were inoculated at once and after having been kept at 34-35° C. for 6 hours, "patient strain" No 8212 being used. All the inoculated samples were incubated for only 9 hours at 34-35° C., after which their toxin content was ascertained. The results of the experiment will be found in table 6.

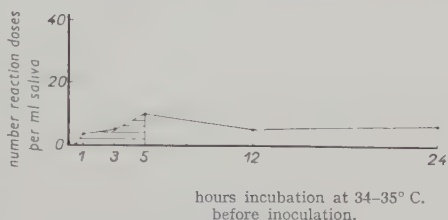


Fig. 3. Toxin production in the saliva of volunteer "v.R.", inoculated at once and after having been kept at 34-35° C. Patient strain No. 7718; incubated for 12 hours at 34-35° C.

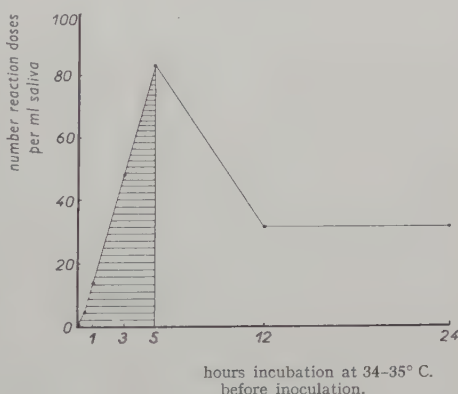


Fig. 4. Toxin production in the saliva of volunteer "v.P.", inoculated at once and after having been kept at 34-35° C. Patient strain No. 7718; incubated for 12 hours at 34-35° C.

TABLE 6.

Formation of diphtheria toxin in saliva of several volunteers, inoculated at once and after 6 hours' incubation at 34-35° C. Patient strain No. 8212; 9 hours' incubation time.

| Saliva sample | Number of "reaction doses" of diphtheria toxin per ml of saliva. |                                                          |
|---------------|------------------------------------------------------------------|----------------------------------------------------------|
|               | Saliva inoculated at once                                        | Saliva inoculated after 6 hours' incubation at 34-35° C. |
| "v.R."        | —                                                                | 10                                                       |
| "v.W."        | —                                                                | 10                                                       |
| "v.Rh."       | —                                                                | 0.5                                                      |
| "v.P."        | —                                                                | 0.9                                                      |

### DISCUSSION.

Our experiments concerning the formation of diphtheria toxin in fresh human saliva have convincingly shown that this fresh saliva may have a strong inhibitory effect on the production of toxin after inoculation with diphtheria bacteria. In this respect they fit in well with the experiments described by DOLD (1940), DOLD and WEIGMANN (1935), BÖNICKE, REIF and ARNDT (1951), FLEMING (1922) and BETHGE, SOEHRING and TSCHESCHE (1947). For the present we will not offer an opinion as to what substance causes this effect. This inhibitory agent is very labile. Not only is it rendered completely inactive when the saliva is autoclaved, but the inhibition is also considerably impaired when the saliva is kept at room temperature or at 34-35° C. for a short time. However, it proved possible to show the presence of diphtheria toxin in nearly every saliva sample we examined, even without any previous treatment, after inoculation with a culture of *C. diphtheriae* and incubation at 34-35° C. For this we used both the well-known non-virulent P.W.8 strain and diphtheria strains freshly isolated from patients. Therefore our conclusion recorded in an earlier publication (TASMAN and SMITH, 1953a) namely that human saliva is a suitable medium for *C. diphtheriae* and that this microbe can, under certain circumstances, produce comparatively large quantities of diphtheria toxin in this saliva, also applies in principle to fresh, non-treated human saliva.

However, the saliva samples examined by us greatly varied as to their capacity to inhibit the production of toxin. It was a striking



fact, that in the various experiments saliva samples obtained from the same persons always showed essentially the same mutual differences. But our material is still too limited to enable us to decide whether we may speak of certain "saliva types" in this respect. It also follows from our studies that the character of the diphtheria strain used will play an important part in this matter.

Bearing all this in mind, we may say with reference to the pathological circumstances of the diphtheria patient, that it will no doubt be possible under certain favourable conditions to show the presence of diphtheria toxin in the saliva of diphtheria patients. This problem is already being investigated as part of a study the results of which will be published in due course.

Related problems may also be seen in the light of these investigations. In this connection we will only raise some questions, without providing an answer.

Is the "carrier-problem", *i.e.* the fact that there are healthy persons who nevertheless harbour and excrete (virulent) diphtheria bacteria without clinically suffering from diphtheria themselves, connected with the "nature" of their saliva? Has their saliva a more potent inhibitory effect than that of non-carriers?

Is there any connection between the types of *C. diphtheriae* we know, *viz.* *gravis*, *mitis* and *intermedius* and the nature of the saliva of the patient in whose throat these strains occur?

Lastly the question arises whether there may be a connection between the results of the above investigations and the occurrence or non-occurrence of "toxic diphtheria".

### S u m m a r y.

In the literature various investigations have been described which point to an evident bactericidal effect of fresh human saliva, especially on *C. diphtheriae*.

In this connection we investigated whether fresh human saliva, when inoculated with *C. diphtheriae*, would inhibit the formation of diphtheria toxin. This actually proved to be the case. The agent responsible for this inhibition of the formation of toxin proved to be extremely labile. The inhibitory effect was completely removed by autoclaving the saliva. But even when the saliva is kept at room temperature, or in the incubator at 34-35° C. for some hours, the inhibitory activity is greatly impaired.

Saliva samples obtained from various volunteers differ greatly in this respect. Some saliva samples show little inhibitory effect, even when very fresh, or lose this capacity very quickly, whereas other samples retain their inhibition for a long time.

This investigation was carried out with the well-known P.W.8 strain and with several diphtheria strains isolated from patients. It could be clearly demonstrated that the strain used has a decisive effect on the toxin formation in saliva.

The conclusion drawn in an earlier publication (TASMAN and SMITH, 1953a), namely that human saliva is a suitable medium for *C. diphtheriae* and that this microbe can, under certain circumstances, form comparatively large quantities of diphtheria toxin in this medium is also applicable to fresh human saliva.

In the light of these results some related problems, connected with the pathogenesis of diphtheria, were discussed.

#### References.

- AMIES, C. R. 1948. J. Pathol. Bact. **60**, 595.  
BERGER, U. 1952. Z.f. Hygiene **136**, 94.  
BETHGE, J., SOEHRING, K. und TSCHESCHE, R. 1947. Z. Naturforschung **2**(b), 12.  
BÖNICKE, R., REIF W. und ARNDT, J. 1951. Z.f. Hygiene **136**, 252.  
DOLD, H. und WEIGMANN, FR. 1935. Z.f. Hygiene **116**, 158.  
DOLD, H. 1940. Z.f. Hygiene **127**, 296.  
FLEMING, A. 1922. Proc. Royal Soc. London, Ser.B. **93**, 306.  
LEVINE, L., WYMAN, L. W. and EDSALL, G. 1949. J. Immunol. **63**, 219.  
TASMAN, A. and SMITH, L. 1953 a. Antonie van Leeuwenhoek **19**, 135.  
TASMAN, A. and SMITH, L. 1953 b. Antonie van Leeuwenhoek **19**, 333.  
THOMPSON, R. and SHIBUYA, M. 1946. J. Bact. **51**, 671.
-

(Chemistry Department, Allahabad University, Allahabad, India).

# A STUDY OF THE INFLUENCE OF AMMONIUM SULPHATE ON THE MINERAL INTAKE OF *PICHLA MEMBRANAEFACIENS* AT DIFFERENT pH

(PRELIMINARY NOTE)

by

S. RANGANAYAKI

(Received February 5, 1954).

## INTRODUCTION

It is a well known fact that the inorganic content of microbial cells differs enormously not only from species to species and from strain to strain (1,2) but also along with the inorganic content of the medium in which the organism is grown. GUILLEMAN and HARSON (3) while studying *B. coli* found that on death about half of the salts diffuses out, whilst the other half remains fixed in the cell and contributes to the amount of the cell ashes. This accounts for the large differences observed in the mineral contents of the different strains of the same species (4), if in one case a living organism and in another case a dead one has been used for analysis. Besides the mineral contents of the living cells can also vary enormously by growing the organism in a same medium at different pH. The more interesting aspect is, that the nature of the influence of this pH on the mineral content of the organism is entirely changed when the quantity of the ammonium sulphate as nitrogen source is changed.

## EXPERIMENTAL.

All the culture media studied in these papers contained 0.2 g NaCl, 0.2 g  $\text{CaCO}_3$ , 0.2 g  $\text{K}_2\text{SO}_4$ , 0.2 g  $\text{Na}_2\text{HPO}_4$  and 0.25 mg  $\text{MgCO}_3$ . These minerals were digested in dilute HCl and the total volume made up to 400 ml. The amounts of  $(\text{NH}_4)_2\text{SO}_4$  as mentioned in the following table were added to each batch, the pH of which

subsequently was adjusted as desired. The media were sterilised and after cooling 14.6 g of absolute ethyl alcohol as a source of carbon were added to each batch. After inoculation with *Pichia membranaefaciens* these cultures were kept for 30 days at room temperature, which varied from 25°-30° C. A good growth was observed. After 30 days yeast cells were filtered off and dried at 30° C. in a dessicator over  $\text{CaCl}_2$ . The dried yeast was powdered and 1 g of each sample was heated in a silica crucible of known constant weight at 350°-450° C. After the material had completely turned to ashes, its weight was determined.

Gram of ashes left on burning one gram of yeast:

| pH  | 1.25 g of $(\text{NH}_4)_2\text{SO}_4$<br>added to the medium |                                               | 2.50 g of $(\text{NH}_4)_2\text{SO}_4$<br>added to the medium |                                               | 3.75 g of $(\text{NH}_4)_2\text{SO}_4$<br>added to the medium |                                               |
|-----|---------------------------------------------------------------|-----------------------------------------------|---------------------------------------------------------------|-----------------------------------------------|---------------------------------------------------------------|-----------------------------------------------|
|     | g of ash<br>left on<br>burning<br>1 g of<br>yeast             | % of<br>yeast<br>yield in<br>the cul-<br>ture | g of ash<br>left on<br>burning<br>1 g of<br>yeast             | % of<br>yeast<br>yield in<br>the cul-<br>ture | g of ash<br>left on<br>burning<br>1 g of<br>yeast             | % of<br>yeast<br>yield in<br>the cul-<br>ture |
| 3   | 0.0172                                                        | 31.20                                         | 0.0175                                                        | 28.30                                         | 0.0067                                                        | 34.71                                         |
| 3.5 | 0.0152                                                        | 26.25                                         | 0.0181                                                        | 28.76                                         | 0.0082                                                        | 30.95                                         |
| 4.0 | 0.0148                                                        | 35.40                                         | 0.0188                                                        | 28.17                                         | 0.0105                                                        | 33.41                                         |
| 4.5 | 0.0105                                                        | 28.65                                         | 0.0198                                                        | 28.55                                         | 0.0173                                                        | 28.53                                         |
| 5.0 | 0.0054                                                        | 32.40                                         | 0.0122                                                        | 31.68                                         | 0.0021                                                        | 35.12                                         |
| 5.5 | 0.0023                                                        | 30.37                                         | 0.0064                                                        | 27.10                                         | 0.0023                                                        | 31.91                                         |

#### DISCUSSION AND SUMMARY.

In the cultures of *Pichia membranaefaciens* containing ethyl alcohol as the only source of carbon and various amounts of  $(\text{NH}_4)_2\text{SO}_4$  as a source of nitrogen, a great diversity in the mineral uptake of the cell is observed. In cultures containing 1.25 g  $(\text{NH}_4)_2\text{SO}_4$  per 400 ml the ashes content regularly decreases with the increase of pH from 3 to 5.5. In the cultures containing 2.5 and 3.75 g  $(\text{NH}_4)_2\text{SO}_4$  the ashes content regularly increases up to pH 4.5.



### References.

1. BAMBOVICEANU, A. and BARKER, C. 1931. Arch. roum Rath. Exp. et Microbiol. **4**, 5.
  2. BAWSON, A. I. and SIA, R. H. P. 1919. J. Bact. **4**, 133.
  3. GUILLEMAN, M. and LARSON, W. P. 1922. J. Inf. Dis. **31**, 355.
  4. FALK, I. S. 1923. Abstr. Bact. **7**, 33.
-



